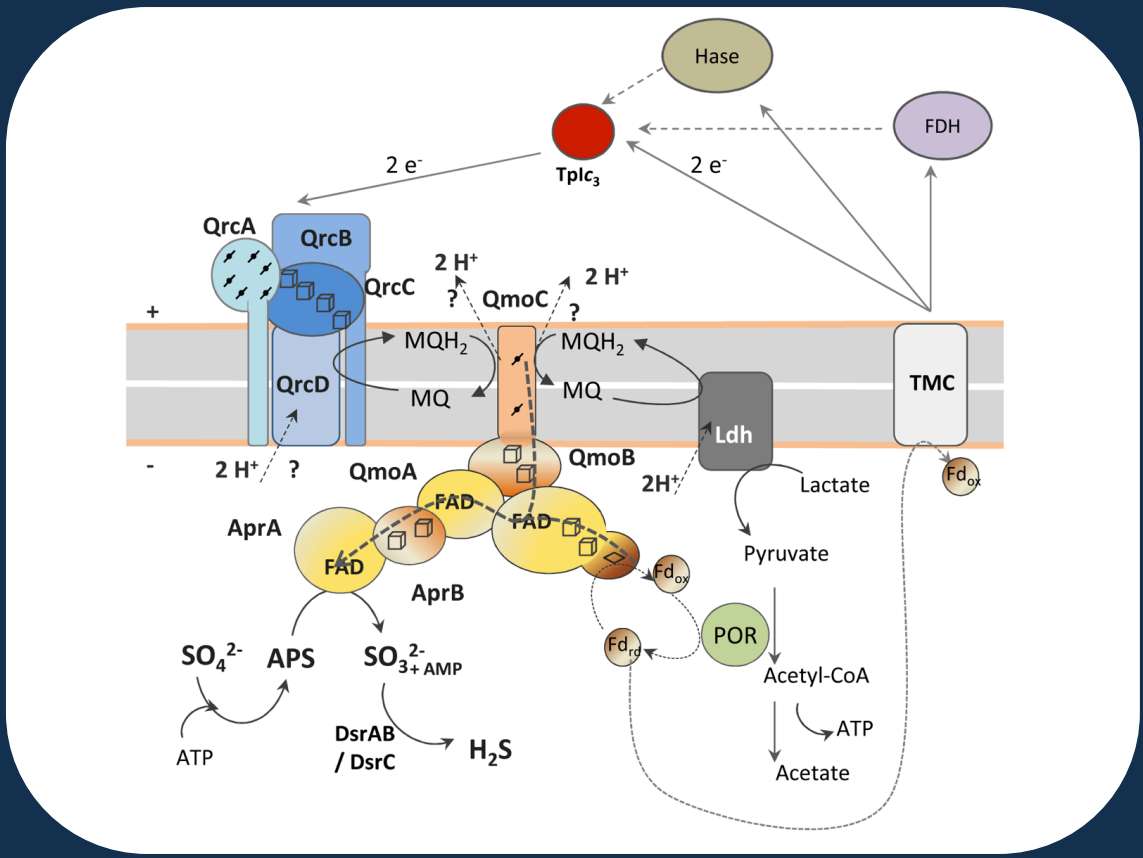
Study of novel energy metabolism pathways in anaerobic bacteria

Ana Raquel Martinho Ramos



Dissertation presented to obtain the Ph.D degree in Biochemistry

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
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Supervisor: Dr. Inês A. C. Pereira

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Prof. Carlos Salgueiro
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From left to right: Uwe Deppenmeier, Wolfgang Nitschke, Ana Raquel Ramos, Inês Pereira, Hermínia de Lencastre, Wolfgang Buckel, Carlos Salgueiro and Manuela Pereira.

July 15, 2014

Bacterial Energy Metabolism Laboratory

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This thesis is dedicated to my parents

THESIS OUTLINE

The work described in this thesis concerns the study of novel energy metabolism pathways in anaerobic bacteria focusing on the investigation of flavin-based electron bifurcation in the energy metabolism of sulfate reducing bacteria.

The thesis starts with an introductory chapter which is divided in two parts. In the first part a description of the mechanism of flavin-based electron bifurcation is presented, with examples of protein complexes that perform this mechanism. The second part describes the principal characteristics of sulfate reducing organisms, with a special attention to *Desulfovibri*o sp. and to proteins that are involved in sulfate reduction. Chapter two describes a genomic analysis to 25 genomes of sulfate reducers, focusing on proteins essential for sulfate reduction, proteins involved in cytoplasmic electron transfer and heterodisulfide reductase-like proteins, as well as a structural and evolutionary insight on proteins involved in dissimilatory sulfate reduction. Chapters three and four describe experimental results obtained during this work. Chapter three consists on the investigation of the physiological role of a conserved membrane-bound complex in sulfate reducing prokaryotes, QmoABC. Chapter four describes a new protein complex from *Desulfovibrio vulgaris*, the Flavin oxidoreductase, which together with Heterodisulfide reductase is involved in ethanol metabolism, possibly through flavin-based electron bifurcation. The last chapter consists of general conclusions and future perpectives of the work.

LIST OF PUBLICATIONS

A comparative genomic analysis of energy metabolism in sulfate reducing bacteria and archaea.
Pereira IAC, **Ramos AR**, Grein F, Marques MC, Marques da Silva S and Venceslau SS
Front. Microbio. (2011) 2:69. doi: 10.3389/fmicb.2011.00069

The membrane QmoABC complex interacts directly with the dissimilatory adenosine 5´-phosphosulfate reductase in sulfate reducing bacteria.
Ramos AR, Keller KL, Wall JD and Pereira IC
Front. Microbio. (2012) 3:137. doi: 10.3389/fmicb.2012.00137

Unifying concepts in anaerobic respiration: Insights from dissimilatory sulfur metabolism.
Grein F, **Ramos AR**, Venceslau SS, Pereira IA.
Biochim. Biophys. Acta. (2013) Feb;1827(2):145-60. doi: 10.1016/j.bbabio.2012.09.001.

The *hdrABC-floxABCD* gene cluster encodes a novel NADH dehydrogenase/heterodisulfide reductase widespread in anaerobic bacteria and involved in ethanol metabolism in *Desulfovibrio vulgaris* Hildenborough
Ramos AR, Grein F, Oliveira G, Venceslau SS, Keller KL, Wall JD, Pereira IAC
(2014) to be submitted

DISSERTATION SUMMARY

Energy conservation in chemotrophic anaerobic bacteria is achieved by two possible processes, substrate level phosphorylation (SLP) and electron transfer phosphorylation (ETP). This second mechanism, also known as respiration, involves chemiosmotic coupling. However, a third mechanism for energy coupling was recently proposed: the flavin-based electron bifurcation (FBEB). The FBEB mechanism is characterized by coupling unfavorable reactions to favorable ones, and it has been demonstrated experimentally in acetogens, methanogens and fermentative organisms. It is also believed that this mechanism was present in the early stages of life as an ancestral mechanism to obtain energy. The protein complexes involved in FBEB are cytoplasmic and contain a flavin cofactor (FMN or FAD), and the reaction can be bifurcating if there are two different electron acceptors or confurcating if there are two different electron donors. A common feature is that one of the electron acceptor/donor is usually ferredoxin (Fd).

Sulfate reducing prokaryotes (SRP) are found ubiquitously in anaerobic environments and are metabolically versatile, capable of metabolizing a wide range of substrates. Despite their environmental importance, the mechanism of energy conservation in sulfate respiration remains to be fully elucidated. Moreover, the occurrence of Heterodissuldide reductase-like proteins (Hdr) in sulfate reducers, especially homologous to HdrA, the flavin containing subunit proposed to carry FBEB in methanogens, suggests that FBEB may also occur in sulfate reducers. Thus, the aim of this work was to investigate the mechanisms of energy

conservation in sulfate reducers that may involve FBEB, and at the same time study the physiological role of two protein complexes, one membrane bound, QmoABC, and another cytoplasmic, HdrABC-FloxABCD.

This work starts with a genomic analysis of 25 available genomes of sulfate reducers and a structural and evolutionary overview of proteins involved in dissimilatory sulfate reduction. The genes coding for all the proteins already identified as directly involved in sulfate reduction are present in all SRO analysed: sulfate transporters, ATP sulfurylase, pyrophosphatase, APS reductase, DsrAB, DsrC, DsrMK and Fd[1]. The Qmo complex is also present in the majority of the organisms, except in *Caldivirga maquiligensis* and in Gram-positive bacteria where the QmoC subunit is missing. We found several proteins related to Hdr of methanogens, in particular HdrA, which points to the occurrence of flavin-based electron bifurcation mechanisms. Additionally, we identified a large number of cytoplasmic hydrogenases, formate dehydrogenases and other proteins as possible candidates for electron bifurcation involving diverse electron donors such as H_2, formate, pyruvate and NAD(P)H. We also identified a new redox protein, the Flavin oxidoreductase (FloxABCD) that together with HdrABC is probably involved in FBEB with NAD(P)H, Fd and DsrC. Thus, it seems that SRO conserve energy with membrane-based chemiosmotic energy coupling,

[1] Rabus, R., T. Hansen and F. Widdel (2006). Dissimilatory Sulfate- and Sulfur-Reducing Prokaryotes. The Prokaryotes, Springer New York: 659-768.

and may also use soluble flavin-based electron bifurcation in alternative pathways.

In the first part of the experimental work, we investigated the physiological role of the membrane complex QmoABC (**Q**uinone interacting **m**embrane-bound **o**xidoreductase). Qmo was proposed to be electron donor to APS reductase, since the *qmo* genes are usually found next to *aprBA* genes. A direct connection between QmoABC and sulfate reduction was established when a *Desulfovibrio vulgaris* Hildenborough mutant lacking the *qmoABC* genes was not able to grow with sulfate, but grew well with sulfite or thiosulfate as electron donor[2]. This demonstrated that the Qmo complex is involved in electron flow between the menaquinone pool and adenosine 5'-phosphosulfate (APS) reduction. However, direct electron transfer between the Qmo complex and Apr could not be detected, which could suggest the involvement of third partners in the process.

The protein-protein interaction studies reported herein provided the first direct evidence that QmoABC interacts with AprBA in *Desulfovibrio* spp. *in vitro* and also *in vivo*. The interaction was characterized as strong but with a transient character, as is typical of electron transfer proteins, and the QmoA subunit was identified as the subunit most involved in the interaction. Since no direct electron transfer between menaquinol reduced Qmo and APS through AprBA was observed, an alternative

[2] Zane, G. M., H. C. Yen and J. D. Wall (2010). "Effect of the deletion of qmoABC and the promoter-distal gene encoding a hypothetical protein on sulfate reduction in *Desulfovibrio vulgaris* Hildenborough." Appl Environ Microbiol **76**(16): 5500-9.

electron transfer mechanisms was proposed. This proposal takes into account the fact that QmoA and QmoB are homologous to HdrA and also that menaquinol ($E^{0'}$ – 75 mV) can probably not serve as sole electron donor to APS reduction ($E^{0'}$ APS/SO_3^{2-} = – 60 mV). Additionally the membrane potential (~150 mV) has to be overcome when transferring electrons from the quinone binding site in QmoC to AprBA in the cytoplasm. The proposal involves a reverse electron bifurcation, i.e. electron confurcation. The electron confurcation mechanism considers that menaquinol and a cytoplasmic reductant with low redox potential (probably Fd) could both donate electrons to the Qmo complex, which would confurcate electrons to the APS reductase. Thus, coupling APS reduction with menaquinone pool oxidation through electron confurcation, could contribute to chemiosmotic energy conservation during sulfate reduction. We investigated possible mechanisms of electron confurcation by *in vitro* assays followed by spectrophotometry or by sulfite quantification, but unfortunately could not obtain evidence for confurcation. We propose future experiments involving the reconstitution of the system in lipossomes.

In the second part of the work we performed a detailed characterization of a new NADH oxidoreductase, the **Fl**avin **ox**idoreductase (FloxABCD) from *Desulfovibrio vulgaris* that is also widespread among anaerobic bacteria. FloxA is composed of a FAD binding domain, a NAD(P)-binding domain and a [2Fe-2S] cluster binding site, and is similar to γ subunit of *Pyrococcus furiosus* soluble Hases (SH) I and II. FloxB is an iron-sulfur protein constituted by a binding site for two canonical [4Fe-4S] centers and also 4 additional cysteines that can bind an additional center. FloxB

share homology with β subunit of *P. furiosus* SH I and II. FloxC is similar to FloxB with a binding domain for two [4Fe-4S] center. Finally, FloxD subunit is similar to MvhD subunit of methanogens, the subunit that binds a [2Fe-2S] cluster and is responsible for electron transfer to HdrABC in *Methanothermobacter marburgensis*. In some organism, like in *D. vulgaris* Hildenborough, the FloxC and FloxD are fused in a single protein (FloxCD).

We investigated the physiological function of *flox-hdr* genes in *D. vulgaris* through the generation of two mutants strains, one with a Ω kanamycin cassette in *hdrC* (IPFG01) that induces the premature termination of the transcription of *hdrC* and the downstream genes of the same transcriptional unit, and another strain lacking the *floxA* gene (IPFG02). In the first strain, we could not detect FloxA confirming that *flox* genes are in the same transcriptional unit of *hdr* genes. Gene and protein expression of wild type cells grown with different electron donors for sulfate or sulfite reduction revealed that *floxA* and *hdrA* are more expressed with ethanol as electron donor. Additionally, the neighbouring gene for an alcohol dehydrogenase (*adh1,* DVU2405) is also highly expressed in the same conditions, but is much more expressed than *hdrA* and *floxA* genes demonstrating that *adh1* is not in the same operon region as the *flox-hdr* genes. Phenotypic characterization of the mutant strains revealed that both mutant strains were unable to grow with ethanol as electron donor for sulfate reduction, while the complemented strain (IPFG03) grew similarly to wild type. In pyruvate fermentation, the two mutant strains produced much lower levels of ethanol than the WT, indicating that in these growth

conditions, FloxABCD is involved in reducing NAD^+ for ethanol production. Our results show that the FloxABCD proteins are involved in ethanol metabolism in *Desulfovibrio vulgaris*. We propose that the FloxABCD-HdrABC complex can perform FBEB coupling Fd reduction with NADH to $DsrC_{ox}$ reduction also with NADH.

Overall, this work contributed to a better understanding of how energy can be conserved in sulfate reducing bacteria, with special attention to a novel mechanism of energy conservation, FBEB, which seems to be widespread among chemotrophic anaerobic bacteria.

SUMÁRIO DA DISSERTAÇÃO

A conservação de energia em bactérias anaeróbias quimiotróficas é obtida através de dois processos, a fosforilação a nível do substrato (FNS) e a fosforilação oxidativa (FO). Este segundo mecanismo também é conhecido como respiração e envolve acoplamento quimiosmótico. Contudo, um terceiro mecanismo para acoplamento energético foi recentemente proposto: a bifurcação de electrões à base de flavinas (BEBF). O mecanismo BEBF é caracterizado pelo acoplamento de reacções desfavoráveis a reacções favoráveis, e foi demonstrado experimentalmente em acetogénicos, metanogénicos e organismos fermentativos. Acredita-se também que este mecanismo esteve presente nas fases primordiais da vida como um mecanismo ancestral para obtenção de energia. Os complexos proteicos envolvidos em BEBF são citoplasmáticos e contêm um co-factor de flavina (FMN ou FAD), e a reacção pode ser classificada de bifurcação se existirem dois aceitadores de electrões diferentes, ou confurcação se houver dois dadores de electrões diferentes. Uma característica comum é que um dos aceitadores/dadores de electrões é normalmente ferredoxina (Fd).

Os procariotas redutores de sulfato (PRS) são omnipresentes em ambientes anaeróbios e são metabolicamente versáteis, sendo capazes de metabolizar uma grande quantidade de substratos. Apesar de sua importância ambiental, o mecanismo de conservação de energia na respiração do sulfato continua por ser completamente elucidado. Além disso, verifica-se nos organismos redutores de sulfato a ocorrência de proteínas semelhantes ás reductases de heterodissulfureto (Hdr), em

particular homólogas à HdrA, a subunidade que contém flavina e é proposta ser responsável pela BEBF em metanogénicos, o que sugere que a BEBF pode também ocorrer em redutores de sulfato. Assim, o objetivo deste trabalho foi investigar mecanismos de conservação de energia em organismos redutores de sulfato (ORS) que possam envolver BEBF, e ao mesmo tempo estudar a função fisiológica de dois complexos proteicos, um membranar, o QmoABC, e outro citoplasmático, o HdrABC-FloxABCD.

O trabalho começa com uma análise genómica de 25 genomas de organismos redutores de sulfato e com uma caracterização estrutural e evolutiva de proteínas envolvidas na redução dissimilatória do sulfato. Os genes que codificam para as proteínas já identificadas como envolvidas diretamente na redução de sulfato estão presentes em todos os ORS analisados: transportadores de sulfato, ATP sulfurilase, pirofosfatase, APS redutase, DsrAB, DsrC, DsrMK e Fd. O complexo Qmo está presente na maioria dos organismos, excepto *em Caldivirga maquiligensis* e em bactérias Gram-positivas a subunidade QmoC está ausente. Foram encontradas várias proteínas relacionadas com Hdr's de metanogénicos, em particular HdrA, que sugere a ocorrência de mecanismos de bifurcação de electrões com flavinas. Além disso, foram também identificados várias hidrogenases citoplasmáticas, formato desidrogenases e outras proteínas como possíveis candidatos para a bifurcação de electrões envolvendo diversos dadores de electrões, tais como H_2, formato, piruvato e NAD(P)H. Identificámos também uma nova proteína redox, a oxidoreductase de flavina (FloxABCD) que, juntamente com a HdrABC está provavelmente envolvida em BEBF com NAD(P)H, Fd

e DsrC. Assim, em OSR a conservação de energia é feita por acoplamento quimiosmótico associado à membrana, ou então em alternativa, por mecanismos envolvendo BEBF.

Na primeira parte do trabalho experimental, investigou-se a função fisiológica do complexo membranar QmoABC (oxidoreductase ligada à membrana que interage com quinonas). O Qmo foi proposto ser o dador de electrões da APS redutase, uma vez que os genes do *qmo* são normalmente encontrados próximos dos genes da *aprBA*. Uma ligação direta entre o QmoABC e a redução de sulfato foi estabelecida quando um mutante sem os genes *qmoABC* de *Desulfovibrio vulgaris* Hildenborough foi incapaz de crescer em sulfato, mas cresceu em sulfito ou tiossulfato como dadores de electrões. Isto demonstrou que o Qmo está envolvido na transferência de electrões entre a menaquinona e a adenosina 5'-fosfosulfato (APS). No entanto, a transferência directa de electrões entre o Qmo e Apr não foi detectada, o que pode sugerir o envolvimento de terceiros elementos no processo.

Os estudos de interacção proteína-proteína aqui reportados forneceram a primeira evidência directa de que o QmoABC interage com a AprBA *in vitro* e também *in vivo* em *Desulfovibrio* spp.. A interacção foi caracterizada como forte, mas com um carácter transiente, tal como é característico de proteínas envolvidas em transferência electrónica, e a subunidade QmoA foi identificada como a subunidade mais envolvida na interacção. Uma vez que não foi detectada nenhuma transferência eletrónica directa entre o Qmo reduzido com menaquinol e APS através de AprBA, foi proposto um mecanismos alternativo de transferência de electrões. A proposta teve em conta o facto de as subunidades QmoA e

QmoB serem homólogas à HdrA, e também que o menaquinol ($E^{0'}$ – 75 mV), provavelmente, não pode ser o único dador de electrões para a redução da APS ($E^{0'}$ APS/SO_3^{2-} = – 60 mV). Além disso, o potencial de membrana (~150 mV) tem de ser superado durante a transferência de electrões do QmoC para AprBA no citoplasma. A proposta envolve uma bifurcação reversa de electrões, ou seja, confurcação de electrões. O mecanismo de confurcação de electrões considera que tanto o menaquinol como um redutor citoplasmático com baixo potencial redox (provavelmente Fd) podem ambos transferir electrões para o Qmo, que por sua vez os transfere para a APS reductase. Assim, o acoplamento da redução de APS com a oxidação do menaquinol através da confurcação de electrões, pode contribuir para a conservação de energia quimiosmótica durante a redução do sulfato. Foram investigados possíveis mecanismos de confurcação de electrões por ensaios *in vitro*, seguidos por espectrofotometria ou por quantificação do sulfito formado, mas infelizmente não foi possível obter evidências para a confurcação. Propomos assim em futuras experiências a reconstituição do sistema em lipossomas.

Na segunda parte do trabalho foi realizado uma caracterização detalhada de uma nova oxidoreductase de NADH, a oxidoreductase de flavina (FloxABCD) de *Desulfovibrio vulgaris*, que está também presente em outras Bactérias anaeróbias. A FloxA é composta de um domínio de ligação FAD, um domínio NAD(P) e um domínio de ligação de um centro [2Fe-2S], e é semelhante à subunidade γ da Hase solúvel (SH) I e II de *Pyrococcus furiosus*. A FloxB é uma proteína de ferro-enxofre constituída por dois possíveis centros [4Fe-4S] e apresenta quatro cisteínas

adicionais que podem ligar um centro adicional. A FloxB é homóloga à subunidade β da SH I e II de *P. furiosus*. A FloxC é semelhante a FloxB com um domínio de ligação para dois centros [4Fe-4S]. Finalmente, a subunidade FloxD é semelhante à subunidade MvhD de metanogénicos, a subunidade que possui um centro [2Fe-2S] e é responsável pela transferência de electrões para a HdrABC em *Methanothermobacter marburgensis*. Em alguns organismos, como em *D. vulgaris* Hildenborough, a FloxC e FloxD estão fundidos numa só proteína (FloxCD).

A função fisiológica dos genes *flox-hdr* em *D. vulgaris* foi investigada através da geração de duas estirpes mutantes, uma com a inserção de uma cassete de Ω canamicina no gene *hdrC* (IPFG01) que induz a terminação prematura da transcrição do *hdrC* e dos genes a jusante da mesma unidade transcripcional; e uma outra estirpe com a delecção do gene *floxA* (IPFG02). Na primeira estirpe, não foi detectado a subunidade FloxA confirmando que os genes *flox* estão na mesma unidade de transcrição de genes *hdr*. A expressão génica e proteica de células wild type (WT) crescidas com diferentes dadores de electrões para a redução do sulfato ou sulfito demonstrou que *floxA* e *hdrA* são mais expressos com etanol como dador de electrões. Além disso, um gene vizinho que codifica uma álcool desidrogenase (*adh1*, DVU2405) também é altamente expresso nas mesmas condições, e é muito mais expresso do que os genes *hdrA* e *floxA* demonstrando que *adh1* não pertence ao mesmo operão dos genes *flox-hdr*. A caracterização fenotípica dos mutantes revelou que ambas as estirpes mutantes não são capazes de crescer com etanol como dador de electrões para a redução de sulfato,

enquanto que a estirpe complementada (IPFG03) cresceu de forma semelhante ao WT. Na fermentação em piruvato, as duas estirpes mutantes produziram níveis muito mais baixos de etanol do que o WT, indicando que nestas condições de crescimento, a FloxABCD está envolvida na redução de NAD^+ para a produção de etanol. Os nossos resultados mostram que a proteína FloxABCD está envolvida no metabolismo de etanol em *Desulfovibrio vulgaris*. Assim, propomos que o complexo FloxABCD-HdrABC pode realizar BEBF acoplando a redução de Fd com NADH à redução de $DsrC_{ox}$ também com NADH.

No seu conjunto, este trabalho contribuiu para uma melhor compreensão de como a energia pode ser conservada em bactérias redutoras de sulfato, com especial atenção para um novo mecanismo de conservação de energia, BEBF, que parece estar difundido entre bactérias quimiotróficas anaeróbias.

LIST OF ABBREVIATIONS

Adh – Alcohol dehydrogenase

ADP – Adenosine diphosphate

AMP – Adenosine monophosphate

Apr – adenosine-5'-phosphosulfate reductase

APS – Adenosine 5´-phosphosulfate

ATP – Adenosine triphosphate

Bcd/Etf – butyryl-CoA dehydrogenase/electron transfer complex

BCIP – 5-bromo-4-chloro-3-indolyl phosphate

Car – caffeyl-CoA reductase

CISM – Complex iron–sulfur molybdoenzymes

CoA – Coenzyme A

CoB-SH – coenzyme B, N-7-mercaptoheptanoyl-L-threonine phosphate

Co-IP – Co-imunoprecipitation

CoM-SH – Coenzyme M, 2-mercaptoethanesulfonate

CoM-S-S-CoB – Heterodisulfide

D. – *Desulfovibrio*

D. desulfuricans – *Desulfovibrio desulfuricans* ATCC 27774

D. vulgaris – *Desulfovibrio vulgaris* Hildenborough

DDM – n-Dodecyl-β-D-maltoside

DMN – 2,3-dimethyl-1,4-naphthoquinone

$DMNH_2$ – 2,3-dimethyl-1,4-naphthoquinol

Dsr – Dissimilatory sulfite reductase

$E^{0'}$ – Standard electrode potential

EDC – N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride

ETP – Electron Transport Phosphorylation

FAD – Flavin Adenine Dinucleotide

FBEB – Flavin Based Electron Bifurcation

Fd – Ferredoxin

Fdh – Formate dehydrogenase

Qmo – Quinone-interacting membrane-bound oxidoreductase

Flox – Flavin oxidoreductase

FMN – Flavin mononucleotide

G20 – *Desulfovibrio alaskensis* G20

Hase – Hydrogenase

Hdr – Heterodisulfide reductase

Hmc – High molecular weight complex

HPLC – High Performance Liquid Chromatography

Hyd – [Fe]-only-hydrogenase

Hyt – NADP-specific bifurcating hydrogenase

INT – iodonitrosotetrazolium chloride

Km – Kanamycin

Ldh – Lactate dehydrogenase

LGT – Lateral Gene Transfer

LUCA – Last Universal Common Ancestor

mBBr – monobromobimane

MFR – methanofuran

MOY – MO basal medium with yeast extract

MQ – menaquinone

MQH_2 – menaquinol

Mtr – methyl-coenzyme M reductase

Mvh – F420 non reducing hydrogenase

NAD^+– nicotinamide adenine dinucleotide

NADH – nicotinamide adenine dinucleotide reduced form

NBT – nitro-blue tetrazolium chloride

Nfn – *N*ADH-dependent reduced *f*erredoxin:*N*ADP^+ oxidoreductase

Nhc – Nine heme cytochrome complex

Nox – NADH oxidoreductase

Nrf – polysulfide reductase

PAGE – Polyacrylamide Gel Electroforesis

P_i – inorganic phosphate

pmf – proton motive force

POR – pyruvate:ferredoxin oxidoreductase

PP_i – inorganic pyrophosphate

PVDF – polyvinylidene difluoride

Qrc – Quinone reductase complex

Rnf – *Rhodobacter* nitrogen fixation

RPG – Robert P. Gunsalus

SLIC – Sequence Ligation Independent Cloning

SLP – Substrate Level Phosphorylation

SOB – Sulfur Oxidizing Bacteria

sp. – specie

spp. - species

SPR – Surface Plasmon Resonance

SRB – Sulfate Reducing Bacteria

SRO – Sulfate Reducing Organism

SRP – Sulfate Reducing Prokaryotes

TBS – Tris buffered saline

TBST – Tris buffered saline Tween 20

Tmc – Transmembrane complex

TMH – Transmembrane Helix

TpIc_3 – Type I cytochrome c_3

TPP – thiamine pyrophosphate cofactor

Tris – tris(hydroxymethyl)aminomethane

UV – Ultraviolet

Vho – methanophenazine-reducing [NiFe] hydrogenase

WT – Wild Type

Latin expressions

e.g. – *exempli gratia*, for exemple

et al. – *et alia*, and other people

etc. – *et cetera*, and other things

i.e. – *id est*, that is to say

Amino acids

Ala	A	Alanine	Leu	L	Leucine
Arg	R	Arginine	Lys	K	Lysine
Asn	N	Asparagine	Met	M	Methionine
Asp	D	Aspartate	Phe	F	Phenylalanine
Cys	C	Cysteine	Pro	P	Proline
Gln	Q	Glutamine	Ser	S	Serine
Glu	E	Glutamate	Thr	T	Threonine
Gly	G	Glycine	Trp	W	Tryptophan
His	H	Histidine	Tyr	Y	Tyrosine
Ile	I	Isoleucine	Val	V	Valine

Chapter 1

Introduction

1.1 - ENERGY CONSERVATION IN ANAEROBIC BACTERIA

Energy is the engine of life and without it living organisms would not exist. The energy conservation mechanisms are the chemical processes by which cells produce ATP, the universal molecular currency of energy, which is then used for the generation of chemical processes, movement, heat generation and transport across membranes (Figure 1.1). In chemotrophic organisms energy conservation is coupled to redox reactions in catabolic pathways. The catabolic pathways can be linear with a constant ATP output, like in aerobic respiration, or can be branched, with several possible alternative electron acceptors, as in anaerobic respiration, where each branch can lead to different ATP gains and thermodynamic efficiency of ATP synthesis. Organisms that live under anaerobic conditions are extremely diverse and versatile, exhibiting a great metabolic diversity as a reflex of their adaptation to different environmental conditions (temperature, pH, salinity, oxygen, electron acceptor, etc.) (Thauer *et al.* 1977; Schmitz *et al.* 2006).

In chemotrophic bacteria, ATP can be produced by two energy conservation mechanisms: substrate-level phosphorylation (SLP), in which ATP is generated from energy-rich intermediates; and oxidative phosphorylation or electron-transfer phosphorylation (ETP), where electron carriers are reoxidized by a terminal electron-acceptor with formation of an electrochemical gradient (ΔpH for protons or ΔpNa for sodium ions) across the cytoplasmic membrane that is used by ATP synthase to produce ATP (Thauer *et al.* 1977). This second mechanism of

energy conservation, oxidative phosphorylation, is also known as respiration and involves chemiosmotic coupling (Mitchell 1961).

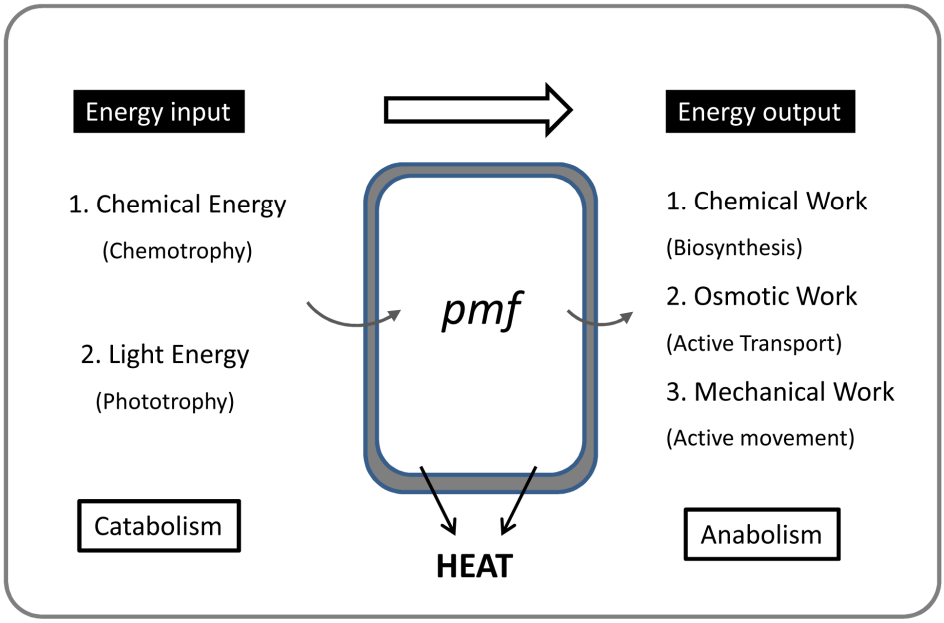


Figure 1.1 - Energetical conversions inside the cell. Adapted from (Thauer *et al.* 1977).

Despite the large availability of carbon substrates there are only a few reactions in anaerobes that conserve energy through SLP (Table 1.1) by comparison to the amount of electron donors/acceptors that can be used to generate energy by ETP (Table 1.2).

Respiratory organisms conserve energy through both pathways, SLP and ETP, using a diverse range of organic and inorganic substrates as electron donors/acceptors in aerobic or anaerobic respiration (Table 1.2) (Thauer *et al.* 1977; Müller 2003; Herrmann *et al.* 2008). Fermentative organisms were classicaly thought to conserve energy exclusively by SLP in a

process where organic compounds (sugars and amino acids) function as both electron donors and acceptors and the excess reductants are removed as reduced compounds (ethanol or H_2, for example).

Table 1.1 - Reactions that yield ATP by substrate-level phosphorylation in anaerobes, adapted from (Schmitz 2006).

Reaction	Enzyme	ΔG_{abs}^{0} (kJ/mol)
1,3-Biphosphoglycerate + ADP ↔ 3-phosphoglycerate + ATP	Phosphoglycerate kinase	–24.1
Phosphoenol pyruvate + ADP ↔ pyruvate + ATP	Pyruvate kinase	–23.7
Acetyl phosphate + ADP ↔ acetate + ATP	Acetate kinase	–12.9
Butyryl phosphate + ADP ↔ butyrate + ATP	Butyrate kinase	–12.9
Carbamoyl phosphate + ADP ↔ carbamate + ATP	Carbamate kinase	–7.5
N-Formyl FH_4 + ADP + P_i ↔ formate + FH + ATP	Formyl-FH_4 synthetase	+8.32
Glycine + $2H^+$ + ADP + Pi ↔ acetate + NH_3 + ATP	Glycine reductase	~ –46.0

FH_4, tetrahydrofolic acid

However, the recent identification of complex chemiosmotic mechanisms in fermentative organisms, such as electrogenic transport in lactic acid bacteria (Lolkema *et al.* 1995), electron transfer through energy conserving hydrogenases in fermentative hyperthermophiles (Sapra *et al.* 2003) or sodium translocating NADH dehydrogenases in glutamate fermenting bacteria (Boiangiu *et al.* 2005), indicate that fermentative organisms are more versatile in the way they conserve energy.

Table 1.2 - Redox potential of electron donors/electron acceptors involved in electron transport phosphorylation, adapted from (Thauer *et al*. 1977; Sato *et al*. 1999).

Redox compound	$E^{0'}$ (mV)	Redox compound	$E^{0'}$ (mV)
SO_4^{2-}/HSO_3^-	– 516	HSO_3^-/HS^-	– 116
CO_2/ formate	– 432	Menaquinone ox/red (MK)	– 74
H^+/H_2	– 414	APS/ AMP + HSO_3^-	– 60
$S_2O_3^{2-}/HS^- + HSO_3^-$	– 402	Crotonyl-CoA/Butyryl-CoA	– 60
Flavodoxin ox/red ($E^{0'}_1$)	– 371	Rubredoxin ox/red	– 57
Ferredoxin ox/red ($E^{0'}_1$)	– 398	Acrylyl-CoA/ propionyl CoA	– 15
NAD^+/NADH	– 320	Glycine/acetate$^-$ + NH_4^+	– 10
Cytochrome c_3 ox/red	– 290	2-Demethylvitamin K_{12} ox/red	+ 25
CO_2/ acetate$^-$	– 290	$S_4O_6^{2-}/S_2O_3^{2-}$	+ 24
S^0/HS^-	– 270	Fumarate/succinate	+ 33
CO_2/CH_4	– 244	Acrylyl-CoA/Propionyl-CoA	+ 60
$FAD/FADH_2$	– 220	Ubiquinone ox/red	+ 113
Acetaldehyde/ ethanol	– 197	$S_3O_6^{2-}/S_2O_3^{2-} + HSO_3^-$	+ 225
Pyruvate$^-$/ lactate$^-$	– 190	NO_2^-/NO	+ 350
$FMN/FMNH_2$	– 190	NO_3^-/NO_2^-	+ 433
Dihydroxyacetone phosphate/ glycerol-phosphate	– 190	Fe^{3+}/Fe^{2+}	+ 772
$HSO_3^-/S_3O_62^-$	– 173	O_2/H_2O	+ 818
Oxaloacetate^{2-}/malate^{2-}	– 172	NO/N_2O	+ 1 175
Flavodoxin ox/red ($E^{0'}_2$)	– 115	N_2O/N_2	+ 1 355

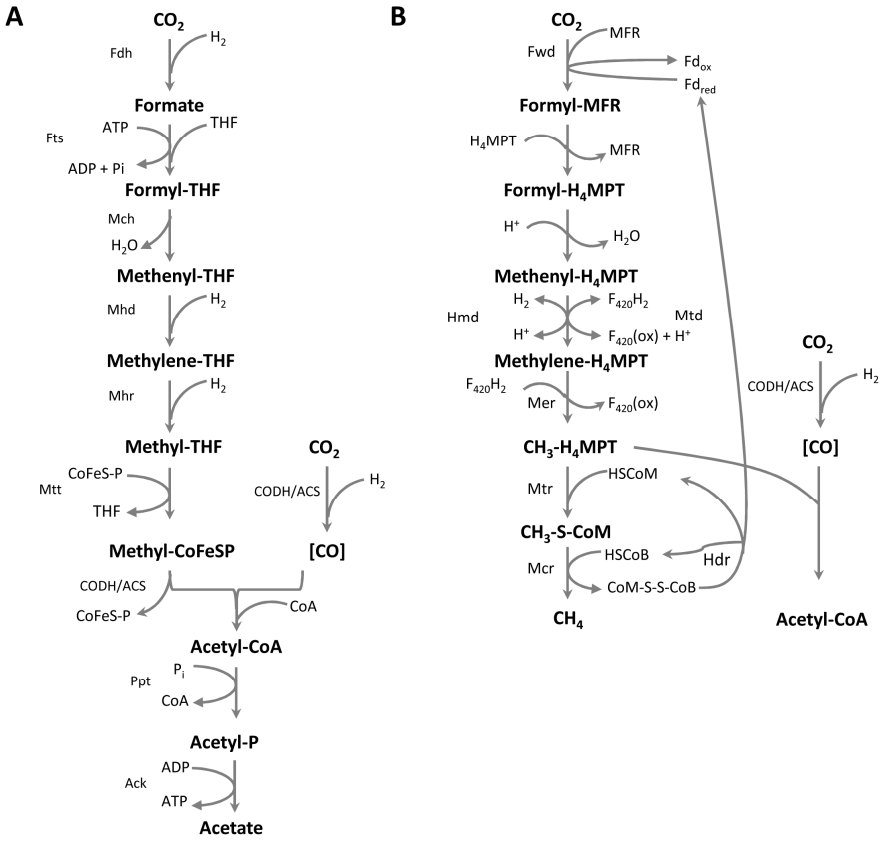
The advent of genomic information in the last years has also provided invaluable information about the evolution of respiratory systems and also of the origin of life itself. In fact, the study of the biology and the processes involved in chemotrophic anaerobic bacteria have contributed for the understanding of how bioenergetic systems may have evolved

(Nitschke and Russell 2009; Martin 2012; Schoepp-Cothenet *et al.* 2013; Sousa *et al.* 2013). In the past, fermentations were believed to be the ancestral mechanisms for energy conservation. But in terms of evolution, enzymes involved in SLP have no signs of antiquity, whereas chemiosmotic coupling systems are found ubiquitously in all living organisms, such as ATP synthase, which was present in the Last Universal Common Ancestor (LUCA) of Bacteria and Archaea (Lane *et al.* 2010; Schoepp-Cothenet *et al.* 2013).

Today, Earth's atmosphere is completely different from the early days of our planet when oxygen was absent, and high temperatures were present together with a highly reducing environment with abundant H_2 and CO_2 (Liu *et al.* 2012; Martin 2012; Poehlein *et al.* 2012; Schoepp-Cothenet *et al.* 2013). Methanogens and acetogens have been proposed to be the most ancestral life forms, as their energy metabolism relies on the electron transfer from H_2 to CO_2, with formation of methane and acetate, respectively (Martin 2012). For this reason methanogens and acetogens are good candidates to study ancestral pathways of energy and carbon metabolism and understand how life forms have evolved within Archaea and Bacteria, respectively (Müller 2003; Liu *et al.* 2012; Martin 2012; Sousa *et al.* 2013). Both archaeal methanogens and acetogenic bacteria reduce CO_2 by the Acetyl-CoA or Wood-Ljungdahl pathway (Scheme 1.1), and both cases present groups that lack cytochromes and quinones (acetogens) or quinone analogs (methanophenazine in methanogens) (Martin 2012).

Quinones are small, liposoluble, proton and electron carriers present in chemiosmotic systems, linking electron donating to electron accepting

enzymes (Simon *et al.* 2008; Schoepp-Cothenet *et al.* 2013). Different kinds of chemiosmotic systems are coupled to different types of quinones, which is also associated with their midpoint redox potential. Generally, quinones can be divided in low-potential carriers such as menaquinone ($E^{0'}$ (MK/MKH_2) = – 70 mV) for anaerobic conditions, and high redox potential quinones like ubiquinone ($E^{0'}$ (UQ/UQH2) = ~+ 100 mV) for aerobic conditions (Simon *et al.* 2008; Schoepp-Cothenet *et al.* 2013). Their redox potential also reflects evolutionary aspects of chemiosmotic mechanisms as the Earth evolved from an ancient anoxic atmosphere to one containing oxygen (Liu *et al.* 2012; Schoepp-Cothenet *et al.* 2013). The absence of quinones or quinone analogs in acetogens and methanogens is unique among autotrophs, and indicates they still conserve energy with chemiosmosis without liposoluble hydrogen carriers (Martin 2012). The first step in methanogenesis and acetogenesis, reduction of CO_2 from H_2 is an endergonic reaction, so how is this reaction possible and at the same time allowing energy conservation? This is a key question in the bioenergetic metabolism of anaerobes, that until recently remained unanswered. The answer to this question was provided by a recent energy metabolism process, described by Buckel, Thauer and co-workers (Herrmann *et al.* 2008; Li *et al.* 2008), named Flavin-based electron bifurcation (FBEB). This process, first described to occur in the homoacetogenic clostridial organism *Clostridium kluyvery* (Li *et al.* 2008), couples an endergonic to an exergonic reaction, where the low redox potential reduced ferredoxin (Fd_{red}) works as energy currency.



Scheme 1.1 - Acetyl-CoA or Wood-Ljungdahl pathway for carbon dioxide fixation and respective enzymes involved in acetogenesis (A) and in methanogenesis (B). Fhd, formate dehydrogenase; Fts, Formyl-THF synthetase; Mch, methenyl-THF cyclohydrolase; Mhd, methylena-THF dehydrogenase; Mhr, methylene-THF reductase; Mtt, methyl-transferase; CODH, CO dehydrogenase; ACS, Acetyl-CoA synthase; Ppt, phosphotransacetylase; Ack, acetate kinase. Fwd, formyl-MFR dehydrogenase; Hmd, H_2-dependent methylene-H_4MPT dehydrogenase; Mtd, F_{420}-dependent methylene-H_4MPT dehydrogenase; Mcr, methyl-CoM reductase; Mtr, methyl-H_4MPT-CoM methyltransferase; Hdr, heterodisulfide reductase. Adapted from (Müller 2003; Costa *et al.* 2010; Poehlein *et al.* 2012).

1.1.1 - ELECTRON BIFURCATION, FLAVOPROTEINS AND FERREDOXIN

The concept of electron bifurcation was first proposed in 1976 by Peter Mitchell to explain the energy conservation in the protonmotive Q-cycle in cytochrome bc_1 (Mitchell 1976). The cytochrome bc_1 complex, also known as Complex III, is present in the mitochondrial inner membrane of eukaryotic cells and in several bacterial electron transfer chains that use oxygen, nitrogen or sulfur compounds as terminal electron acceptors. It is also part of the photosynthetic purple bacteria electron transfer chain and belongs to the larger family of *bc*-type complexes that includes the cytochrome *bf* complex present in chloroplasts, algae and some gram-positive bacteria (Trumpower 1990; Brandt 1996b; Hunte *et al.* 2003). The general complex is composed of three electron transfer proteins: a cytochrome *b* subunit with two heme *b* groups (one with a low redox potential - b_L, and the other with a high redox potential - b_H); a cythocrome c_1 and a $[2Fe\text{-}2S]^{2+/1+}$ Rieske protein (Figure 1.2). In the protonmotive Q cycle, ubiquinol oxidation is linked to proton release in the positive side (center P) of the membrane and ubiquinone is reduced in the negative side (center N) of the membrane with proton uptake. Electron bifurcation takes place at center P during ubiquinol oxidation with electron flow into the high redox potential Rieske $[2Fe\text{-}2S]^{2+/1+}$ cluster ($E^{0'}$ = + 290 mV; exergonic reaction) coupled to the low redox cytochrome b_L reduction ($E^{0'} \approx -$ 20 mV; endergonic reaction) by the ubisemiquinone radical. This electron bifurcation allows vectorial proton translocation across the membrane that contributes to energy

conservation in mitochondrial and many bacterial respiratory chains – Figure 1.2 (Brandt 1996a; Brandt 1996b).

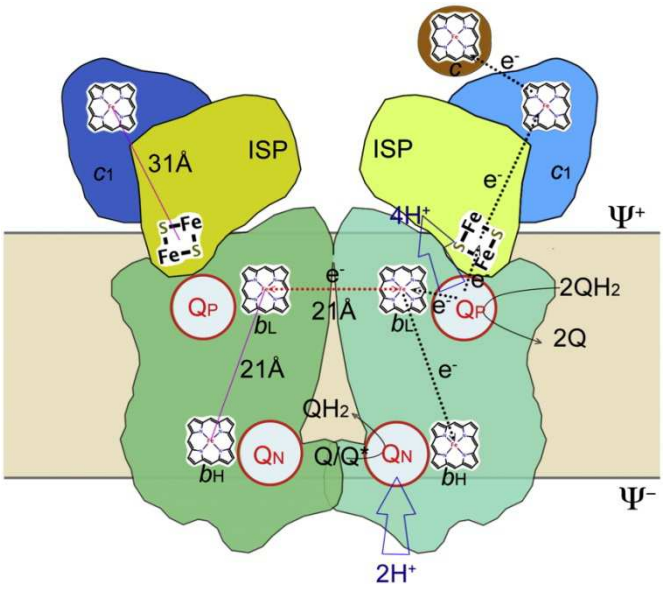


Figure 1.2 - Homodimeric organization of the cytochrome bc_1 complex with representation of the proton-motive Q-cycle mechanism. Ubiquinol is oxidized at center P (Q_P site) and one electron goes to the Rieske Fe-S protein (ISP) generating a low potential ubisemiquinone anion, which immediately reduces heme b_L and subsequently heme b_H with two protons being released at the P site of the membrane. At the same time the electron transferred to the iron-sulfur protein is transferred to cytochrome c_1 and then to cytochrome *c*. The Q-cycle is complete when a second ubiquinol is oxidized in center P and the electrons transferred to b_H end up reducing the ubisemiquinone radical at Q_N with proton uptake from the cytoplasm and ubiquinol release. One complete Q-cycle requires one ubiquinol molecule oxidation at Q_P site, two cytochrome c molecules reduced, four H^+ release at P site and two H^+ uptake at N site. From (Xia *et al.* 2013).

The FBEB mechanism was proposed by similarity to the electron bifurcation that takes place in the center P of the mitochondrial cytochrome bc_1 complex. But in FBEB, flavin cofactors are responsible for the bifurcation reaction in which the formation of a low redox potential flavin semiquinone ("hot flavosemiquinone") radical can be responsible for ferredoxins reduction. Flavins are biological cofactors derived from riboflavin (vitamin B_2) and they are constituted by a redox-active isoalloxazine ring system capable of one electron transfer in two steps or two electron transfers at once. The flavin cofactors found in enzymes, which are called flavoproteins or flavoenzymes, can be classified as flavin mononucleotide (FMN) or flavin-adenine dinucleotide (FAD), according to the group attached to the reactive ring (Figure 1.3). These kind of proteins are very important in biological systems because they are involved in diverse flavin dependent reactions, such as, dehydrogenations, oxidations, monooxygenations, halogenations, reductions and biological sensing (Macheroux *et al.* 2011). Additionally, if combined with other redox-active centers, like iron-sulfur clusters ($[2Fe\text{-}2S]^{2+/1+}$, $[3Fe\text{-}4S]^{1+/0}$ and/or $[4Fe\text{-}4S]^{2+/1+}$), they can be involved in more complex electron transfer reactions, as we can confirm from FBEB reactions.

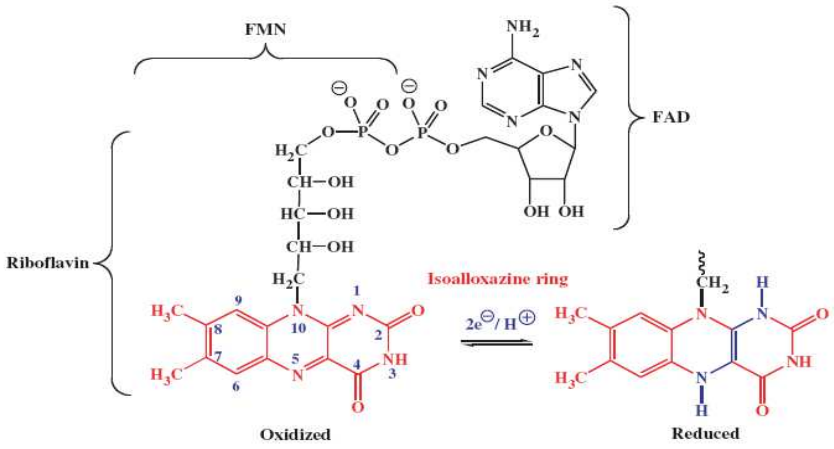


Figure 1. 3 - Representation of riboflavin, FMN and FAD structures, in which the isoalloxazine ring is responsible for the redox activity of the cofactor. The isoalloxazine ring is represented in the oxidized and in the two electron reduced state. From (Macheroux *et al.* 2011).

Ferredoxins (Fd) are cytoplasmic iron-sulfur proteins found in all living organisms from archaea and bacteria to higher plants and animals. These acidic, electron transfer proteins contain one $[2Fe\text{-}2S]^{2+/1+}$ cluster or one, two or more $[4Fe\text{-}4S]^{2+/1+}$ clusters (Sticht and Rosch 1998; Buckel and Thauer 2013). The redox potential of Fd is very low ($E^{0'}$ = – 450 mV), and inside cells they are more than 90% reduced, which makes them good electron donors in reactions with redox potentials as low or even below – 500 mV. They are involved in several processes such as hydrogen metabolism, nitrogen and CO fixation, and nitrite and sulfite reduction among others (Sticht and Rosch 1998). But the most interesting aspect of Fds is their antiquity reflected in their widespread occurrence, which supports the hypothesis that these proteins were

present since the primordial forms of life (Figure 1.4) (Eck and Dayhoff 1966; Kim *et al.* 2012; Sousa *et al.* 2013).

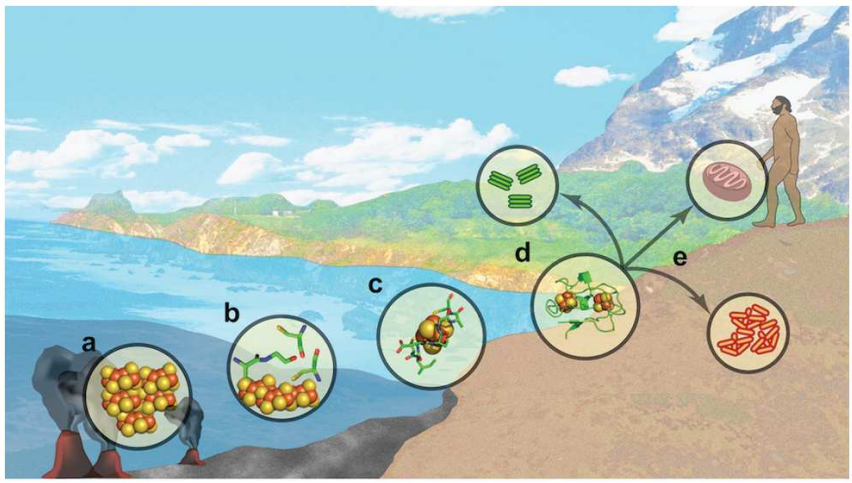


Figure 1.4 - Hypothesized iron-sulfur cluster evolution from hydrothermal vents to life. a) Iron-sulfur minerals such as pyrite (FeS_2) could spontaneously catalyze carbon fixation to generate essential organic molecules for life; b) the organic molecules or amino acids formed allows new chemistry or enhances the existing reactions; c) synthesis of small polypeptides occurs at the water-mineral interface; d) small polypeptides constitute ferredoxin-like proteins; e) ferredoxin is preserved in all forms of life constituting a large domain of redox proteins necessary for life. From (Kim *et al.* 2012).

1.1.2 - THE FLAVIN BASED ELECTRON BIFURCATION MECHANISM

The Gram-positive bacterium *Clostridium kluyvery* is unique among clostridia as it can grow anaerobically on ethanol and acetate as sole energy sources (Seedorf *et al.* 2008). The energy metabolism of *C. kluyvery* has been the object of several studies to try to understand

how the organism ferments ethanol and acetate to butyrate, caproate and H_2, according to the equation:

(1.1) 6 ethanol + 3 acetate$^-$ $\rightarrow$ 3 butyrate $^-$ + caproate $^-$ + H^+ + 4 H_2O + 2 H_2

($\Delta G^{0'}$ = – 183 kJ/mol)

An important question regarding the *C. kluyvery* metabolism is how does it produce H_2?

In 2008, the work of Herrmann *et al.* and Li *et al.* (Herrmann *et al.* 2008; Li *et al.* 2008) could explain the mechanism of H_2 formation from reduced Fd and NADH, in this anaerobic bacterium. Their work demonstrated that H_2 formation was Fd-dependent for reduction of crotonyl-CoA to butyryl-CoA; additionally H_2 was generated during fermentation from NADH in an endergonic reaction:

(1.2) NADH + H^+ $\rightarrow$ H_2 + NAD^+ ($\Delta G^{0'}$ = 20 kJ/mol)

Herrmann *et al.* suggested that the highly exergonic reaction of crotonyl-CoA reduction ($E^{0'}$ = – 10 mV) with NADH ($E^{0'}$ = – 320 mV) could be involved in energy conservation in this organism. During butyrate synthesis, crotonyl-CoA is reduced to butyryl-CoA and this reaction is NADH dependent (Herrmann *et al.* 2008). The work of Li *et al.* (Li *et al.* 2008) demonstrated that Fd reduction ($E^{0'}$ = – 410 mV) with NADH was coupled to reduction of crotonyl-CoA to butyryl-CoA with NADH. The reaction is catalyzed by the cytoplasmic butyryl-CoA

dehydrogenase/electron transfer complex (BcdA/EtfBC complex) – Figure 1.5, in the following reaction:

(1.3) $Fd_{ox} + 2\ NADH + crotonyl\text{-}CoA \rightarrow Fd_{red}^{2-} + 2\ NAD^{+} + butyryl\text{-}CoA$

$(\Delta G^{0\prime} = -44\ kJ/mol)$

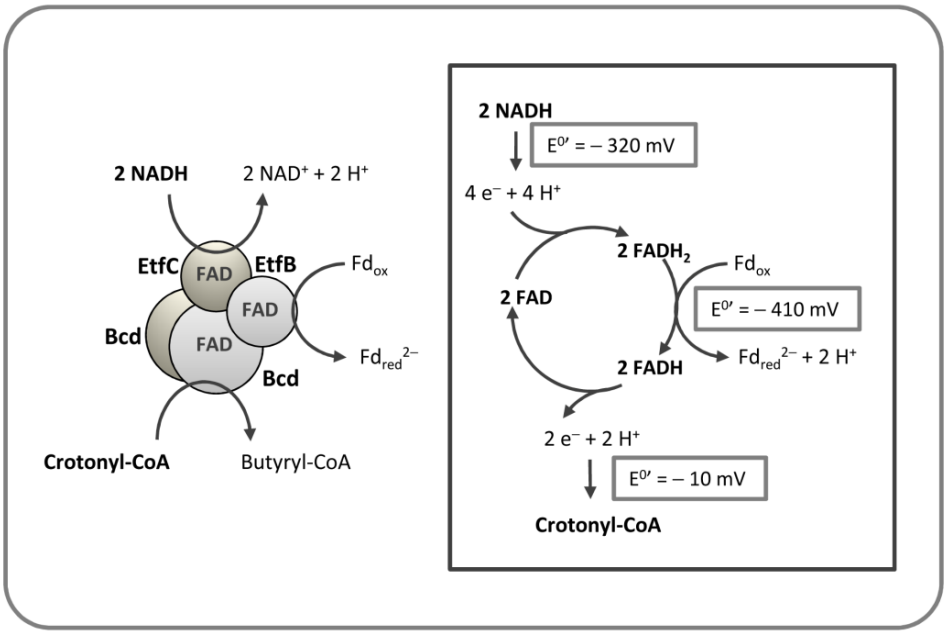


Figure 1.5 - Flavin based electron bifurcation mechanism by Bcd/EtfCB from *Clostridium kluyveri*. The exergonic reduction of crotonyl-CoA by NADH is coupled to the endergonic reduction of ferredoxin with NADH. Adapted from (Li *et al.* 2008).

The BcdA/EtfBC complex contains four FAD cofactors and no other prosthetic group, which is why the investigators named the bifurcation as flavin-based, as FAD is probably involved. Flavins are two electron carriers that in some cases can be reduced by one electron to a stable semiquinone flavin radical ($FADH^{\bullet}$ or $FMNH^{\bullet}$), which can be further

reduced to the fully reduced flavin nucleotide ($FADH_2$ or $FMNH_2$). Generally the first one electron reduction has a much more positive redox potential than the second electron reduction (Li *et al.* 2008). The Bcd/Etf protein complex is currently one of the best well studied examples of FBEB, and the mechanism of electron bifurcation has been characterized through structural, biochemical, spectroscopic and kinetic studies (Chowdhury *et al.* 2014). In a more recent study with Bcd/Etf from *Acidaminococcus fermentans*, Chowdhury and coworkers proposed the mechanism in Figure 1.6. The reaction starts with a two electron transfer, in the form of hydride, from NADH to β-FAD in Etf generating β-$FADH^-$ [$E^{0'}$ (β-FAD/β-$FADH^-$) = – 280 mV] (Figure 1.6A and 1.6B). The electron bifurcation in β-$FADH^-$ could generate a one electron transfer with a redox potential downhill to α-FAD generating α-$FAD^{\bullet-}$ (– 60 mV), accompanied of domain reorientation, and the remaining β-$FADH^{\bullet}$ with a low redox potential (– 500 mV) reduces Fd (Figure 1.6C and 1.6D). The domain position change in Etf prevents the more favorable reduction of α-$FAD^{\bullet-}$, and instead the electron is transferred to the D-FAD in Bcd. In the second cycle, Figure 1.6E-F-A, Fd is reduced and D-$FADH^-$ generated in Bcd is used for crotonyl-CoA reduction to butyryl-CoA (Chowdhury *et al.* 2014). This concerted mechanism is similar to what happens in the $[2Fe\text{-}2S]^{2+/1+}$ Rieske protein in Complex III of the mitochondrial respiratory chain. Structural studies have demonstrated that movement of the iron-sulfur center is necessary for the bifurcation reaction to be possible, coupling the unfavorable reaction to the favorable one in the Q cycle (Xia *et al.* 2007; Xia *et al.* 2013).

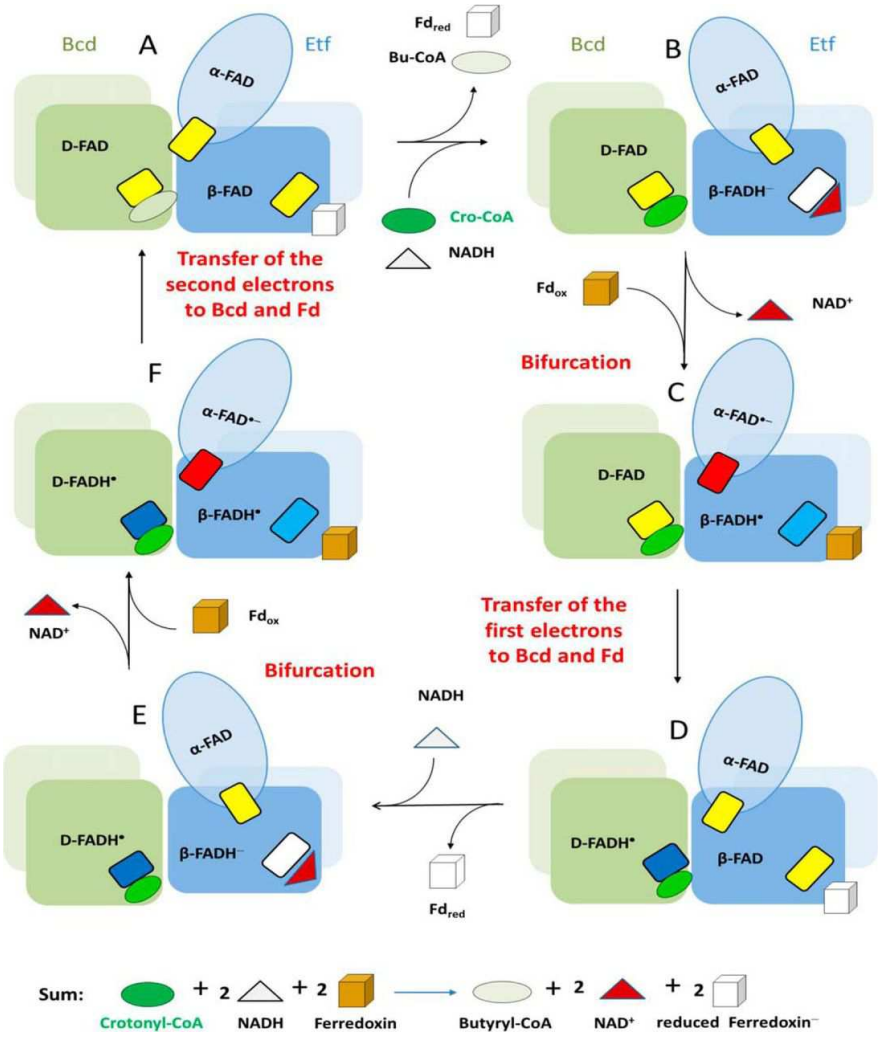


Figure 1. 6 - Mechanism of flavin-based electron bifurcation proposed to operate at the Bcd/Etf complex based on structural considerations. Bcd dimers are represented interacting with Etf domains. The small rectangules represent FAD: yellow at the quinone state (FAD), red at the anionic semiquinone state ($FAD^{\bullet -}$), light blue and dark blue at the neutral semiquinone state ($FADH^{\bullet}$) and in white at the hydroquinone state ($FADH^{-}$). From (Chowdhury *et al.* 2014).

Since the reaction components are not directly involved in proton or ion gradients, how can FBEB be considered a mechanism of energy conservation? The Fd_{red} can be considered as energy currency and function like ATP plus NAD(P)H, contributing to energy conservation in acetogenic organisms by two possible routes: by reducing protons to H_2 increasing SLP in the oxidative branch of fermentation or by generating an electrochemical gradient via the Rnf membrane complex (Herrmann *et al.* 2008). The Rnf complex was first discovered in *Rhodobacter capsulatus* and since the genes were involved in nitrogen fixation the complex was named Rnf for ***R****hodobacter* **n**itrogen **f**ixation (Rnf). Rnf is an H^+/Na^+-pumping ferredoxin:NAD^+ oxidoreductase that can be found in many anaerobes (see (Biegel *et al.* 2011) for a review).

Soon after the first publication regarding the FBEB mechanism in *Clostridium*, a review article about energy conservation in methanogens proposed that FBEB would also operate in methanogens that also lack cytochromes (Thauer *et al.* 2008).

1.1.2.1 - FBEB AND METHANOGENS

In methanogens, methane formation from CO_2 reduction with H_2 is coupled to the formation of heterodisulfide (CoM-S-S-CoB) in a reaction catalyzed by methyl-coenzyme M reductase (Mtr). The heterodisulfide functions as the terminal electron acceptor of an energy-conserving electron transport chain, and its reduction mechanism is distinct in methanogens with and without cytochromes. Heterodisulfide reductase from methanogens with cytochromes is composed of two subunits: one

membrane bound subunit with hemes *b* (HdrE) and a soluble iron-sulfur containing subunit (HdrD) (Ide *et al.* 1999; Deppenmeier and Müller 2008). HdrDE receives electrons from the methanophenazine pool through heme *b,* which are then conducted to HdrD where heterodisulfide reduction takes place. HdrDE together with the membrane methanophenazine-reducing [NiFe] hydrogenase (VhoACG) contributes to energy conservation during methanogenesis (Deppenmeier 2004; Hedderich *et al.* 2005; Thauer *et al.* 2008) (Figure 1.7A).

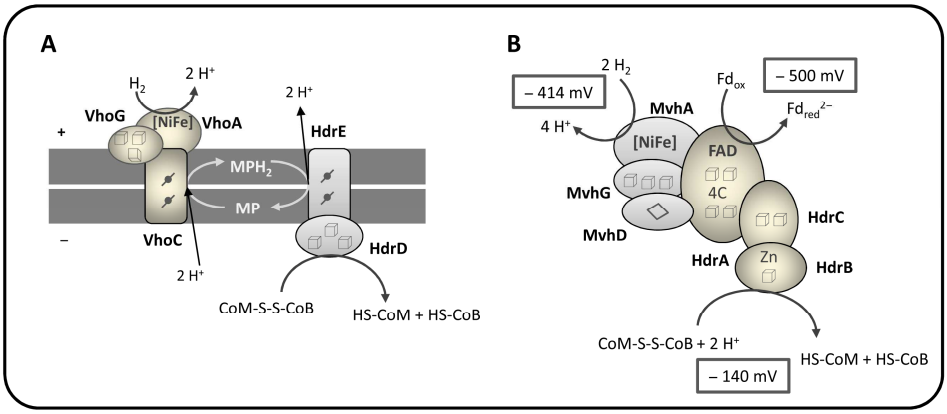


Figure 1.7 - Schematic representation of heterodisulfide reduction and coupled reactions in methanogens. (A) HdrDE/VhoACG complex from cytochrome-containing methanogens, in which heterodisulfide reduction is coupled to chemiosmosis. Adapted from (Thauer *et al.* 2010). (B) HdrABC/MvhADG complex from methanogens that lack cytochromes. The complex is responsible for heterodisulfide reduction with H_2 while coupling it to an endergonic reaction, Fd reduction by H_2, in a FBEB mechanism. Adapted from (Thauer *et al.* 2008). Cubes – [4Fe-4S] clusters; diamond – [2Fe-2S] cluster; - heme b.

In methanogens that lack cytochromes heterodisulfide reductase is a soluble protein complex composed of three subunits: HdrB with one [4Fe-4S]$^{2+/1+}$ cluster, HdrC containing two [4Fe-4S]$^{2+/1+}$ clusters, with both subunits sharing homology with HdrD (which is like a hypothetical fusion protein of HdrB and HdrC); and HdrA that contains four [4Fe-4S]$^{2+/1+}$ clusters and one FAD (Hedderich *et al.* 2005; Thauer *et al.* 2008). HdrABC forms a tight complex with F_{420}-non-reducing hydrogenase (MvhADG) and together they are responsible for heterodisulfide reduction by H_2 (Figure 1.7B).

The catalytic subunits in heterodisulfide reductases are HdrB in hydrogenotrophic methanogens and HdrD in methylotrophic methanogens. The subunits HdrA and HdrE are most likely the contact points with the physiological electron donors. In the case of membrane-associated HdrDE, electron transfer from methanophenazine is linked to energy conservation in heterodisulfide reduction, but HdrA is a cytoplasmic protein with no membrane association of any kind, so how is heterodisulfide reduction coupled to energy conservation in this situation?

The answer to this question relies in the FBEB mechanism proposed by Thauer and co-workers (Thauer *et al.* 2008) to operate in hydrogenotrophic methanogens, by similarity to the Bcd/Etf complex of clostridia. Since HdrA is a flavin containing protein, the two electron carrier FAD could bifurcate electrons from H_2 ($E^{0'}$ = – 414 mV) for heterodisulfide reduction ($E^{0'}$ = – 140 mV), and to Fd reduction ($E^{0'}$ = – 500 mV). The flavin based mechanism was demonstrated by the work of (Kaster *et al.* 2011), in which the whole complex was isolated and CoM-S-

S-CoB reduction by H_2 was shown to be dependent on Fd reduction by H_2:

$$(1.4) \quad Fd_{ox} + 2H_2 + CoM\text{-}S\text{-}S\text{-}CoB \rightarrow Fd_{red}^{2-} + CoM\text{-}SH + CoB\text{-}SH + 2H^+ \quad (\Delta G^{0'} = -55 \text{ kJ/mol})$$

The mechanism in Figure 1.7B can explain a long observed effect (RPG effect) (Gunsalus and Wolfe 1977). Costa and coworkers (Costa *et al.* 2010; Costa *et al.* 2013b), also proved by protein-protein interaction studies that in hydrogenotrophic methanogens heterodisulfide reductase (Hdr), formylmethanofuran dehydrogenase (Fwd), F_{420}-nonreducing hydrogenase (Vhu) and formate dehydrogenase (Fdh) can interact *in vivo* and thus in methanogenesis, H_2 or formate can be used as electron donors and conserve energy by FBEB, and that the first and last step of methanogenesis are physically connected (RPG effect). The energy is conserved because the exergonic heterodisulfide reduction is coupled to reduction of Fd, which is then used for the endergonic reduction of CO_2 to formylmethanofuran (formyl-MFR) (Scheme 1.1). The fundamental part of this mechanism is HdrA that is responsible for electron bifurcation and generation of low-potential electrons. Costa *et al.* demonstrated that either with H_2 or formate, electrons flow from hydrogenases or formate dehydrogenases through VhuD (homologous to MvhD) to HdrA, where bifurcation takes place. HdrA and MvhD are fused in some species stressing that MvhD subunit is involved in electron transfer from an electron donor to HdrA (Hedderich *et al.* 2005). Another important characteristic is that HdrA is one of the most highly conserved

proteins in methanogens, but it is also found in non-methanogens like sulfate reducers, suggesting that this bifurcation mechanism can also be found in other contexts beyond methanogenesis (Stojanowic *et al.* 2003; Buckel and Thauer 2013; Costa *et al.* 2013b).

1.1.2.2 - THE BIFURCATING [FEFE]-HYDROGENASES

Right after the publication of the first FBEB mechanism performed by BcdA-EtfBC other FBEB protein complexes were characterized. In common they have Fd-dependent reactions and the presence of FAD or FMN cofactors. One of these proteins was isolated from the hyperthermophilic *Thermotoga maritima*, the heterotrimeric [FeFe] hydrogenase (HydABC), which coupled the oxidation of Fd ($E^{0'}$ = – 453 mV) and oxidation of NADH ($E^{0'}$= – 320 mV) to generate H_2 ($E^{0'}$ = – 420 mV) – Figure 1.8A (Schut and Adams 2009):

(1.5) $NADH + 2Fd_{red}^{2-} + 3H^+ \leftrightarrow 2H_2 + NAD^+ + 2Fd_{ox}$

($\Delta G^{0'}$ = + 21 kJ/mol)

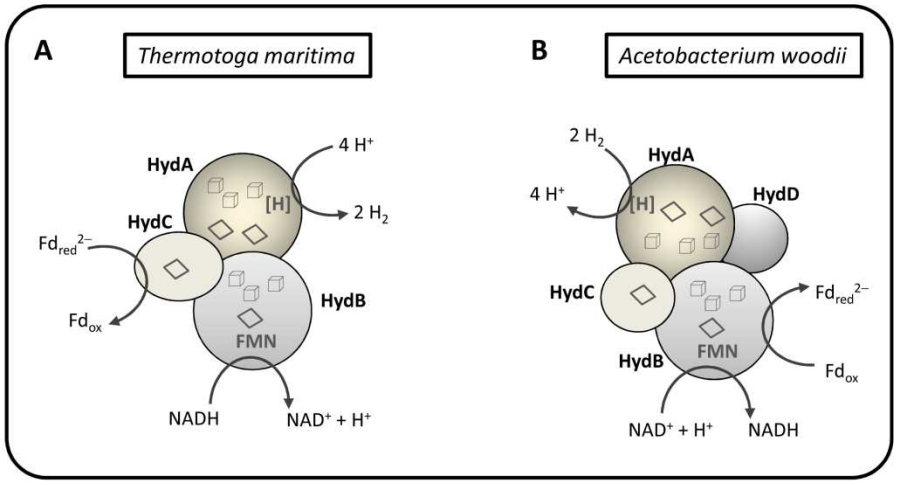


Figure 1.8 - Schematic representation of the structure and function of two electron bifurcating [FeFe] hydrogenases. (A) HydABC from *Thermotoga maritima* is responsible for coupling Fd_{red} and NADH oxidation in a reverse flavin-based electron bifurcation (confurcation) reaction for H_2 generation. Adapted from (Schut and Adams 2009). (B) HydABCD from *Acetobacterium woodii* couples Fd reduction by H_2 with NAD^+ reduction by H_2 in a FBEB reaction. Adapted from (Schuchmann and Müller 2012). Cubes – [4Fe-4S] cluster; diamonds – [2Fe-2S] cluster.

HydABC is a trimeric protein with subunits in a 1:1:1 ratio; HydA is predicted to contain three $[4Fe\text{-}4S]^{2+/1+}$ clusters, two $[2Fe\text{-}2S]^{2+/1+}$ clusters and the H cluster where H_2 is formed; HydB is predicted to bind three $[4Fe\text{-}4S]^{2+/1+}$ and one $[2Fe\text{-}2S]^{2+/1+}$ clusters and the FMN cofactor; HydC is supposed to bind one $[2Fe\text{-}2S]^{2+/1+}$ cluster (Buckel and Thauer 2013).

In the energy metabolism of *T. maritima* glucose is fermented with generation of NADH and Fd_{red}, whose oxidation in turn must be coupled to H_2 formation. The mechanism of H_2 production was solved with the characterization of the bifurcating FMN-containing hydrogenase, HydABC, that was proven to couple oxidation of Fd and NADH to H_2

generation through a reverse flavin based electron bifurcation, also named as confurcation (Schut and Adams 2009) (Figure 1.8A).

Another electron-bifurcating hydrogenase was isolated and characterized from the acetogenic bacterium *Acetobacterium woodii* (Schuchmann and Müller 2012). *A. woodii* is a model organism to study acetogenesis without cytochromes and by extension to understand ancient metabolisms, as it contains only one site for electrochemical ion gradient generation (sodium-motive ferredoxin: NAD^+-oxidoreductase, the Rnf complex). The HydABCD complex was purified and characterized and it was demonstrated that the [FeFe]-hydrogenase uses electron bifurcation. The HydABC from *T. maritima* shares similarity to the bifurcating hydrogenase subunits from *A. woodii*. The endergonic reduction of Fd by H_2 could be explained due to the presence of FBEB performed by the HydABCD complex that couples this reaction to the exergonic reduction of NAD^+ by H_2 (Figure 1.8B). A similar bifurcating [FeFe]-hydrogenase was also isolated and characterized from *Moorella thermoacetica* (Wang *et al.* 2013a), that performs the coupled Fd and NAD^+ reduction with H_2.

A novel type of NADP-specific bifurcating hydrogenase (HytA-E) was isolated from *Clostridium autoethanogenum* grown on CO. This hydrogenase forms a functional complex with a formate dehydrogenase (FdhA) (Wang *et al.* 2013a). Similarly to methanogens (Costa *et al.* 2010; Costa *et al.* 2013a; Costa *et al.* 2013b), this bifurcating hydrogenase can perform electron bifurcation with H_2 or formate as electron donors. The complex was shown to perform the reversible reaction, coupling both reduction of Fd and $NADP^+$ with H_2 or formate, and in the reverse

reaction generate formate from H_2 and CO_2 (Wang *et al.* 2013a), presenting the two functions *in vivo*. This double function is important to understand alternative energy metabolism pathways that organisms seem to adopt as a way to overcome energetically challenging growth conditions.

In *Clostridium acidurici* an electron-bifurcating formate dehydrogenase (HylCBA-FdhF2) was also reported to couple NAD^+ and ferredoxin reduction with formate by FBEB in the uric acid metabolism of this acetogen (Wang *et al.* 2013b). This was the first bifurcating formate dehydrogenase identified and for sure many more will be identified in the future.

1.1.2.3 - OTHER EXAMPLES OF FBEB ENZYMES

A cytoplasmic bifurcating protein complex from *C. kluyveri* was isolated and characterized right after the Bcd-Etf complex (Wang *et al.* 2010). The transhydrogenase NfnAB (**N**ADH-dependent reduced **f**erredoxin:**N**ADP^+ oxidoreductase), is an iron-sulfur flavoprotein that couples the exergonic $NADP^+$ reduction with Fd_{red} to the endergonic reduction of $NADP^+$ with NADH in a reverse electron bifurcation mechanism also called electron confurcation – Figure 1.9A (Huang *et al.* 2012), similarly to the bifurcating hydrogenase in *T. maritima.* The reversible reaction occurs as follows:

$$(1.6) \quad NADH + Fd_{red}^{2-} + 2NADP^+ + H^+ \leftrightarrow NAD^+ + Fd_{ox} + 2NADPH$$
$$(\Delta G^{0'} = -20 \text{ kJ/mol})$$

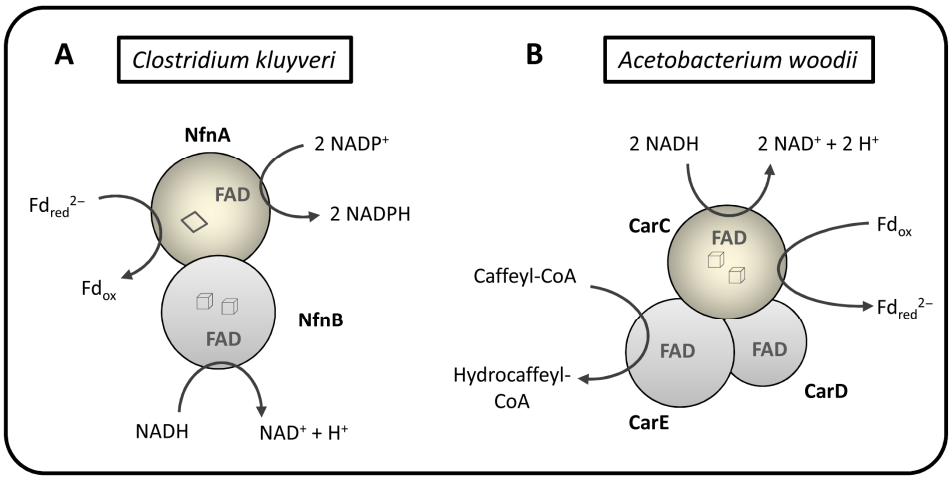


Figure 1.9 - Schematic representation of structure and function of: (A) NfnAB from *Clostridium kluyveri* that couples the exergonic $NADP^+$ reduction with Fd_{red} and the endergonic reduction of $NADP^+$ with NADH in a reversible reaction. Adapted from (Buckel and Thauer 2013). (B) Caffeyl-CoA reductase complex (CarCDE) from *Acetobacterium woodii* couples the endergonic Fd reduction by NADH with the exergonic caffeyl-CoA reduction by NADH during caffeate respiration. Adapted from (Bertsch *et al.* 2013). Cube – [4Fe-4S] cluster; diamond – [2Fe-2S] cluster.

NfnAB from the acetogen *Moorella thermoacetica* has also been isolated and characterized presenting similar properties of the complex from *C. kluyveri* (Huang *et al.* 2012). In both cases, NfnA and NfnB were heterologously expressed in *E. coli*, purified and characterized. NfnA contains a $[2Fe\text{-}2S]^{2+/1+}$ cluster and a FAD cofactor, while NfnB contains two $[4Fe\text{-}4S]^{2+/1+}$ clusters and FAD. When separated the proteins do not perform reaction (1.6) but when mixed in a 1:1 ratio they are active (Buckel and Thauer 2013).

More recently, an electron-bifurcating caffeyl-CoA reductase was isolated and characterized from *A. woodii* cells grown in fructose

(Bertsch *et al.* 2013). In this acetogenic organism caffeate respiration can be used to produce Fd_{red} that can be oxidized by the Rnf complex to generate a Na^+-transmembrane gradient that drives ATP synthesis via $Na^+F_1F_0$-ATP synthase (Heise *et al.* 1991; Reidlinger *et al.* 1994). In addition to the bifurcating Hase previously described (Schuchmann and Müller 2012), caffeyl-CoA reductase-Etf complex (CarCDE) bifurcates electrons coupling the endergonic reduction of Fd with NADH to the exergonic reduction of caffeyl-CoA with NADH, contributing to the energy conservation in *A. woodii* (Figure 1.9B).

1.1.2 - IMPORTANT CONSIDERATIONS ON FBEB MECHANISMS

All archaeal and bacterial protein complexes involved in FBEB have in common that they are cytoplasmic flavoproteins (FMN or FAD containing) and they can use simultaneously two different electron acceptors when the reaction is bifurcating or two electron donors when the reaction is confurcating, with one of the electron acceptor/donor being Fd (Buckel and Thauer 2013; Wang *et al.* 2013a). The flavin cofactor seems crucial in the FBEB mechanism and two mechanisms were proposed to explain how bifurcation happens. One mechanism, proposed by Buckel and Thauer, is based on the redox potentials that flavoproteins exhibit in their three possible redox states: $E^{0'}$ (FP/FPH_2) for the fully reduced state (2 electrons), $E^{0'}$ (FP/FPH) and $E^{0'}$ (FPH/FPH_2) for one electron reduction states (1 electron). They assume that the flavosemiquinone state is stable and that $E^{0'}$ (FP/FPH) is in general more positive and $E^{0'}$ (FPH/FPH_2) more negative than (FP/FPH_2). The

bifurcation of the two electrons in FPH_2 promotes one electron reduction of two different one-electron acceptors: one electron acceptor with low redox potential (Fd, endergonic reaction) and the other with a much more positive redox potential (exergonic reaction) (Kaster *et al.* 2011; Schuchmann and Müller 2012). Structural information and enzymatic activity are crucial to establishing the mechanism of FBEB, as it was proven by the recent work on the Bcd/Etf complex from *Acidaminococcus fermentans* (Chowdhury *et al.* 2014). The flavin-based bifurcation mechanism in Bcd-Etf complex takes into account the stable intermediary flavoquinone plus conformational changes during the electron transfer that enables the endergonic reaction to take place instead of the exergonic one. Nitschke and Russel proposed a different mechanism, based on crossed-over redox potentials. In this proposal flavins do not have a stable semiquinone state and the fully reduced flavin (FPH_2) is first involved in an exergonic reaction, reducing the "high potential electron" acceptor, generating an unstable "hot flavosemiquinone" with a redox potential low enough to reduce ferredoxin. The reaction is possible because both electron acceptors and flavin cofactor are in close proximity (Nitschke and Russell 2011; Schuchmann and Müller 2012). Both mechanisms seem to be dependent of the distances between the electron donors/acceptors, so it is important to study the protein structures of all complexes that have been reported to perform FBEB.

Another important aspect in studying this type of reactions regards the evolutionary aspects related to quinone-based and flavin-based electron bifurcations. Ubiquinone-based electron bifurcations are membrane

associated reactions that take place at redox potentials close to that of ubiquinone (+ 110 mV), while flavin-based bifurcations are associated to cytoplasmic enzyme complexes and have much lower redox potentials (– 200 mV) (Huang *et al.* 2012). As we know, Earth evolved from an atmosphere with a low redox potential to one with a much more positive redox potential, so electron bifurcation reactions may reflect also how organisms evolved together with Earth's atmosphere (Sousa *et al.* 2013). We must also consider the increasing number of protein complexes that carry FBEB that have been characterized in the last few years, and likely many more will appear in the near future, proving that this mechanism is very widespread among anaerobes (Martin 2012; Buckel and Thauer 2013).

1.2 - THE SULFUR CYCLE AND SULFATE REDUCING BACTERIA

The Earth's biogeochemical cycles are important for living processes since life started to evolve ~4 billion years ago. Microorganisms, especially Bacteria and Archaea, participate in the global cycling of several chemical elements such as sulfur, nitrogen, carbon and iron. They are able to use a variety of organic and inorganic compounds as electron donors or terminal electron acceptors to generate energy in the form of ATP (Hedderich *et al.* 1998; Madsen 2011).

Sulfur is one of the most abundant elements on Earth. Sulfur can be found mainly in three forms: elemental sulfur (S^0; oxidation state 0), sulfate (SO_4^{2-}; fully oxidized state +6) and sulfide (S^{2-}; fully reduced state −2) (Tang *et al.* 2009; Simon and Kroneck 2013). Microorganisms are able to convert sulfur compounds from one state to another generating the microbial sulfur cycle (Figure 1.10) (Tang *et al.* 2009).

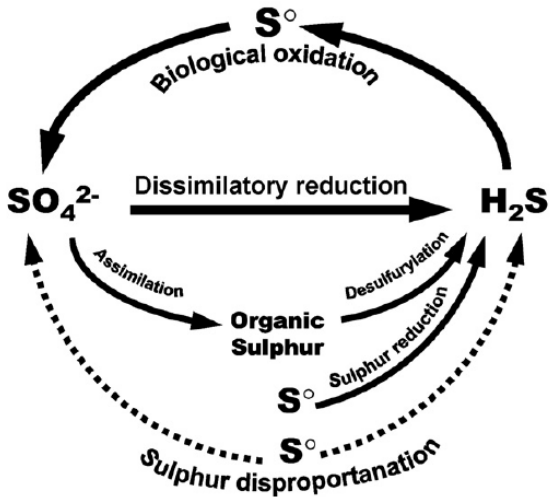


Figure 1. 10 - Microbial sulfur cycle, from (Tang et al 2009).

Microorganism that use sulfate as the terminal electron acceptor, sulfate reducing organisms (SRO), play an important role in the global sulfur cycle, presumably for more than 3.5 billion years ago as supported from fractionation of stable sulfur isotopes (Shen and Buick 2004). Thus sulfate reducers, together with methanogens and acetogens, are apparently ancestral organisms on Earth. The study of sulfate reducers and their fundamental metabolic pathway, sulfate reduction ($SO_4^{2-} \rightarrow SO_3^{2-} \rightarrow S^{2-}$), is important to understand how these organisms operate and, more importantly, to develop their biotechnological applications (Hockin and Gadd 2007; Muyzer and Stams 2008; Barton and Fauque 2009).

1.2.1 - PHYLOGENETIC AND PHYSIOLOGICAL DIVERSITY OF SRB

Sulfate reducers are widespread in the environment and are metabolically versatile. These microorganisms, mostly Bacteria and a few Archaea (Figure 1.11), can be found in anoxic environments like marine and fresh waters, sediments, soil, and also in the mouth and gut of several animals, including humans (Matias *et al.* 2005; Muyzer and Stams 2008). They are particularly abundant in marine sediments because of the high content of sulfate (~ 28 mM) in sea water (Canfield *et al.* 2000), and are responsible for more than 50% of total carbon mineralization in these sediments (Jorgensen and Fenchel 1974; Jorgensen 1982).

The first sulfate reducing bacterium isolated, because of its ability for sulfide production from sulfate, was named *Spirillum desulfuricans* due to its morphology (Beyerinck 1895; Rabus *et al.* 2006), and later was

reclassified as *Desulfovibrio desulfuricans*. For more than 80 years sulfate reducing bacteria (SRB) were classified according to phenotypic characteristics and up until the 1980's the number of species identified was limited. But the development of genomic, genetic and biochemical tools contributed to a more detailed classification of sulfate reducers. Since the early 1980's species started to be classified based on 16S ribosomal RNA (rRNA), based on reverse transcriptase sequencing of 16S rRNA in the late 1980's, and from 1990 PCR-mediated amplification and sequencing techniques were applied to SRB (Thauer *et al.* 2007; Muyzer and Stams 2008).

Figure 1.11 shows a phylogenetic tree of SRO based on 16S rRNA, grouped in seven phyla, five within Bacteria and two in Archaea. The majority of sulfate reducers belong to the *Deltaproteobacteria* division, followed by the *Clostridia* with the Gram-positive SRO (*Desulfotomaculum*, *Desulfosporosinus* and *Desulfosporomusa* genera). Thermophilic SRO are grouped in three lineages: *Nitrospirae* (*Thermodesulfovibrio* genus), *Thermodesulfobacteria* (*Thermodesulfobacterium* genus) and *Thermodesulfobiacea* (*Thermodesulfobium* genus). Finally the archaeal sulfate reducers belong to the *Euryarchaeota* (*Archaeoglobus* genus) and to the *Crenarchaeota* lineage (*Thermocladium* and *Caldivirga* genera) (Muyzer and Stams 2008; Barton and Fauque 2009).

Another phylogenetic technique is based on the analysis of functional genes that encode key enzymes of sulfur metabolism such as the dissimilatory sulfite reductase (*dsrAB*) or the adenosine-5'-phosphosulfate reductase (*aprBA*). These genes are conserved in all SRO

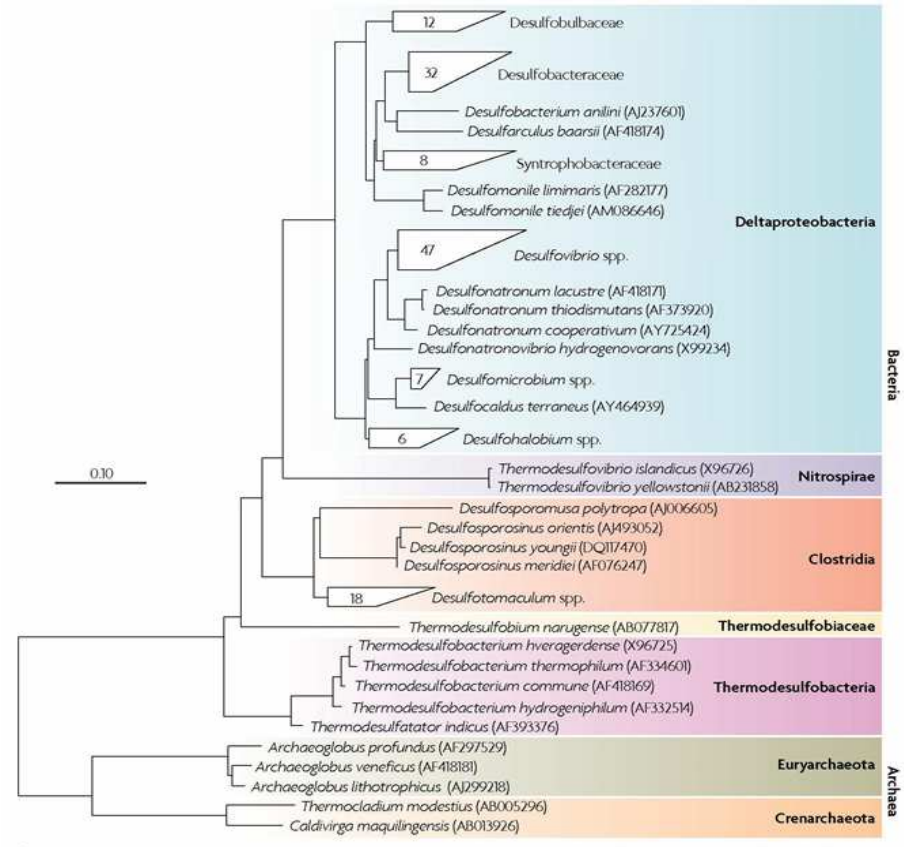


Figure 1.11 - Phylogenetic tree based on 16S ribosomal RNA (rRNA) sequences of described SRO described. From Muyzer and Stams 2008.

and indicate a mostly vertical evolution from a common ancestor, with a few episodes of lateral gene transfer (LGT) events (Wagner *et al.* 1998; Teske *et al.* 2003; Meyer and Kuever 2007; Muyzer and Stams 2008).

SRB are capable of sulfate reduction with a variety of electron donors like hydrogen, lactate, pyruvate or ethanol. Other possible electron donors are sugars (e.g. fructose, glucose), amino acids (e.g. glycine, serine, alanine), monocarboxylic acids (acetate, propionate and butyrate), dicarboxylic acids (fumarate, malate and succinate), alcohols

(methanol, ethanol, etc) and aromatic compounds (e.g. benzoate and phenol) (Rabus *et al.* 2006; Barton and Fauque 2009). The major end product of sulfate reduction is sulfide, and normally carbon compounds are incompletely oxidized to acetate (Table 1.3), but some organisms can completely oxidize them to CO_2, commonly in marine organisms. Incomplete oxidation occurs when organisms lack the mechanism for terminal oxidation of acetyl-CoA (Rabus *et al.* 2006; Muyzer and Stams 2008). Sulfide can be found in different forms (S^{2-}, H_2S or HS^-) according to environmental pH, and can be oxidized back to elemental sulfur or sulfate by sulfur oxidizing bacteria (chemotrophic or phototophic). The production of hydrogen sulfide is responsible for the negative impact of SRB in the environment as it is a highly corrosive and flammable gas with a characteristic smell (Rabus *et al.* 2006; Muyzer and Stams 2008; Tang *et al.* 2009).

Sulfate is not the only terminal electron acceptor of SRB, which can also use other sulfur compounds like sulfite (SO_3^{2-}), thiosulfate ($S_2O_3^{2-}$) or sulfur (S^0). Additionally other electron acceptors can be used by some species, such as nitrate and nitrite that are reduced to ammonium; and several metal ion oxides including iron (Fe^{III}), uranyl (U^{VI}), selenate (Se^{VI}), chromate (Cr^{VI}) and arsenate (As^{VI}) can be used by sulfate reducers (Muyzer and Stams 2008). This capacity of sulfate reducers makes them suitable for bioremediation of toxic metals. Some sulfate reducers are also able to conserve energy through microaerobic respiration, proving that they are not strictly anaerobic but can survive low concentrations of O_2 (Cypionka 2000; Dolla *et al.* 2006; Sass and Cypionka 2007).

Table 1.3 - Oxidation of various electron donors coupled to sulfate reduction and the corresponding Gibbs free energy. Adapted from (Liamleam and Annachhatre 2007; Tang *et al.* 2009).

Reaction	ΔG^0 (kJ/reaction)
Hydrogen: $4H_2 + SO_4^{2-} \rightarrow 4\ H_2O + S^{2-}$	– 123.98
Acetate: $CH_3COO^- + SO_4^{2-} \rightarrow H_2O + CO_2 + HCO_3^- + S^{2-}$	– 12.41
Formate: $4HCOO- + SO_4^{2-} \rightarrow 4\ HCO_3^- + S^{2-}$	– 182.67
Pyruvate: $4CH_2COCOO^- + SO_4^{2-} \rightarrow 4CH_3COO^- + 4CO_2 + S^{2-}$	– 331.06
Lactate: $2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2CO_2 + H_2O + S^{2-}$	– 140.45 or – 178.06
Malate: $2(OOCCH_2CHOHCOO)^{2-} + SO_4^{2-} \rightarrow 2CH_3COO^- + 2CO_2 + 2HCO_3^- + S^{2-}$	– 180.99
Fumarate : $2\ (OOCCHCHCOO)^{2-} + SO_4^{2-} + 2H_2O \rightarrow 2CH_3COO^- + 2CO_2 + 2HCO_3^- + S^{2-}$	– 190.19
Succinate: $4(OOCCH2CH2COO)^{2-} + 3SO_4^{2-} \rightarrow 4CH_3COO^- + 4CO_2 + 4HCO_3^- + 3S^{2-}$	– 150.48
Ethanol: $2CH_3CH_2OH + SO_4^{2-} \rightarrow 2CH_3COO^- + 2H_2O + S^{2-}$	– 133

This ability of sulfate reducers reflects also adaptation to their habitats, because many sulfate reducers live in close proximity to oxic/anoxic interfaces (Jorgensen 1977; Hoehler *et al.* 1994; Muyzer and Stams 2008). Finally, sulfate reducers are also capable of growing in the absence of terminal electron acceptors by fermenting organic acids (e.g. malate, fumarate, pyruvate, lactate, sugars) and alcohols (ethanol); or grow in lactate or ethanol syntrophically with other organisms like hydrogenotrophic methanogens (Walker *et al.* 2009; Morris *et al.* 2013).

1.2.2 - THE GENUS *DESULFOVIBRIO*

Desulfovibrio species are among the most abundant SRO genus within the *Deltaproteobacteria* but are also the most studied genus biochemically, physiologically and genetically, due to its rapid and easy growth. *Desulfovibrio* are non-sporulating Gram-negative bacteria, mesophilic with optimal temperatures of growth between 25 – 40 °C, motile with a single polar flagella, curved or rod shaped with sizes between 0.5 - 1.3 × 0.8 - 5 µm and an optimal pH between 6.6 – 7.5. These bacteria can be found preferentially in anoxic freshwaters and marine sediments, but also in oil fields, industrial water systems and in animals intestines (Holt 1994). *Desulfovibrio* sp. use hydrogen, organic acids or alcohols as electron donors for dissimilatory sulfate reduction coupled to electron transport phosphorylation.

In the last decade several genomes of SRO were sequenced and deposited on databases and can be accessed on genome websites such as MicrobesOnline (www.microbesonline.org), the Integrated Microbial Genomes (IMG, http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) and the Genome database from the National center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/sites/genome). Currently there are 40 *Desulfovibrio* spp. sequenced genomes available in the IMG website. *D. vulgaris* Hildenborough (hereafter *D. vulgaris*)*, D. alaskensis* G20*, D. desulfuricans* ATCC 27774 (hereafter *D. desulfuricans*) and *D. gigas* are the more common species used by researchers to study the physiology and the biochemistry of sulfate reducers. The work

performed for this thesis was done with *D. vulgaris* and *D. desulfuricans* species.

D. vulgaris was isolated in 1946 from clay soil near Hildenborough, Kent, United Kingdom (Postgate 1984); it is a model organism for SRB studies and it was the first SRB to have its genome sequenced (Heidelberg *et al.* 2004). *D. vulgaris* has been used successfully in genetic manipulations like chromosomal insertions and deletions through homologous recombination, and plasmid insertion and replication (Chhabra *et al.* 2011; Keller *et al.* 2011). These methods are a starting point for genetic manipulation of other sulfate reducers (Rabus *et al.* 2006; Keller *et al.* 2011). *D. desulfuricans* is also used in genetic manipulations, because like *D. vulgaris* it has a high plating efficiency and antibiotic sensitivities suitable for mutant selection. It was the first Deltaproteobacterium to undergo transduction (Voordouw and Wall 1993; Rabus *et al.* 2006). The genome of *D. desulfuricans* ATCC 27774 has also been recently sequenced; this strain was isolated from the gastrointestinal tract of a sheep by Marvin Bryant and was characterized as a sulfate-reducing bacterium capable of growing with dissimilatory nitrate reduction (Bryant 1977; Marietou *et al.* 2009). This strain was also used for biochemical isolation and characterization of membrane-associated electron transport complexes (Pires *et al.* 2003; Matias *et al.* 2005; Pires *et al.* 2006a).

1.2.3 - THE IMPACT OF SRB METABOLISM

The metabolism of SRB has positive and, negative consequences for the environment. The reason for the negative impact of sulfate reducers metabolism lies in the end product, sulfide. In the middle of the twentieth century, the economical and environmental impact of sulfate reduction was recognized, mainly due to the toxicity and corrosive properties of hydrogen sulfide (Barton and Fauque 2009). The sulfide produced and released is the principal responsible for biocorrosion, pollution, souring of oil and gas reservoirs and is also implicated in health problems.

Corrosion of iron pipes and support structures has a great economical impact, particularly for the oil industry, and for countries in general due to corrosion of sewage pipes and drinking water supply systems. Aerobic corrosion is a chemical process, while the anaerobic corrosion is linked to the activity of microorganisms, also called microbially-influenced corrosion (MIC) or biocorrosion, and is frequently related to the activity of SRB (Dinh *et al.* 2004). Since the majority of bacteria responsible for biocorrosion are sulfate reducers, this group of bacteria are the most investigated in this area, principally because SRB inhabit a large range of anoxic habitats with a variety of nutrients, temperatures, pressures and pH values (Beech and Sunner 2007). Sulfate reducers when associated with microbial consortia have the ability to form biofilms in metallic surfaces generating anoxic zones where SRB can develop. These biofilms are responsible for the biocorrosion, because metal ions when in contact

with sulfide form metal sulfide products that precipitate when in contact with the anoxic zone (Beech and Sunner 2007).

Biocorrosion and oil souring are the principal economical consequences of SRB in the petroleum industry, as well as the toxicity of sulfide for workers in the oil fields. Sulfide can be as toxic as cyanide when inhaled in high concentration and may be fatal (above 500 parts per million), but exposure to low concentrations can also have consequences for humans originating eye and mucous membrane irritation (EPA 2003; Caffrey and Voordouw 2010). Souring is the biological production of hydrogen sulfide in crude oil *in situ*, and is a consequence of the presence of microorganisms that have the capacity to live in such extreme environments that are oil reservoirs. SRB are among the organisms that can be isolated from oil reservoirs and their presence is of great concern because of damage due to sulfide production (Magot *et al.* 2000; Ollivier *et al.* 2007). The metabolical variety of SRB allows their survival in oil reservoirs as they have available a variety of electron donors and sulfate as acceptor, due to the use of sea water as makeup water, and SRB have the capacity to degrade crude oil (Magot *et al.* 2000). The effects of the activity of SRB in oil reservoirs can be controlled with addition of nitrate and biocides. The nitrate stimulates nitrate-respiring bacterium and the nitrites produced inhibit sulfate respiration reducing the oil souring (Haveman *et al.* 2005). In combination with nitrite, the use of biocides like glutaraldehyde, formaldehyde, bronopol, among others, have proven to be effective in controlling the activity of SRB in the biofilms (Greene *et al.* 2006).

Another area of concern regarding SRB is the health of human beings. SRB are not only found in the environment but they are also present in the digestive tract of animals and humans. They are part of the microbiota ecosystem of the human large intestine and of animal guts and therefore contribute for the host physiology and metabolism (Loubinoux *et al.* 2002b; Macfarlane *et al.* 2007). Although they are not considered significant pathogens they have been associated to disease such as inflammatory bowel disease (Crohn's disease and ulcerative colitis) (Pitcher and Cummings 1996; Loubinoux *et al.* 2002b; Rowan *et al.* 2009) and also periondontitis (Langendijk *et al.* 2001; Loubinoux *et al.* 2002a).

The SRB that inhabit the human large intestine belongs to the genera *Desulfovibrio, Desulfobacter, Desulfotomaculum* and *Desulfobulbus*. The presence of a higher content of SRB, especially *Desulfovibrio* spp., in faeces of patients with inflammatory bowel disease, compared to healthy individuals, suggests that sulfate reducers have a role, or are associated with the inflammation (Gibson *et al.* 1991; Loubinoux *et al.* 2002b). Three species have been isolated from patients with inflammatory bowel diseases: *D. piger*, *D. desulfuricans*, *D. fairfieldensis*, with a prevalence of *D. piger* in this kind of illness (Loubinoux *et al.* 2002b). The human faecal hydrogen sulfide content is in the range of 0.3 - 3.4 mmol/l, and a higher content, due to an imbalance of the microbiota, can be toxic and cause inflammation in the intestinal epithelium. Sulfide can affect DNA integrity and ATP dependent potassium channels and can inhibit butyrate oxidation in colonocytes, the principal source of energy for these cells, causing chronic

inflammation and cell death (Pitcher and Cummings 1996; Rowan *et al.* 2009). Other diseases related to the digestive tract have been related to *Desulfovibrio* spp., such as cholecystitis and abdominal abcesses, and also some forms of colorectal cancer can be caused by sulfide formation, which can activate biochemical pathways that in turn can cause the disease (Macfarlane *et al.* 2007).

Besides the large intestine, SRB can also be found in the oral microbiota. The more common sulfate reducers *D. fairfieldensis* and *Desulfomicrobium orale* have been isolated from periodontal pockets and are associated to the pathogenesis of periodontitis, with higher prevalence of *Desulfovibrio* spp. (Langendijk *et al.* 2001; Loubinoux *et al.* 2002a). In a recent study, *D. desulfuricans* was also isolated from human saliva, and the presence of SRB was detected in the oral microbiota of healthy individuals (Heggendorn *et al.* 2013). A high prevalence of SRB was also associated to patients with gastritis and periodontitis, this last one directly related to the presence of SRB (Heggendorn *et al.* 2013). In addition, *Desulfovibrio* spp. have also been reported to be implicated in brain abscesses, blood stream infections and in gynecological or obstetric pathologies (e.g. *D. intestinalis* isolated from the vaginal flora) (Macfarlane *et al.* 2007; Ichiishi *et al.* 2010).

Although high levels of hydrogen sulfide can be lethal, low levels can provide beneficial effects and regulate physiological functions in humans. Recent studies demonstrated that H_2S has a therapeutic potential for mammalian regulation, as found previously for nitric oxide or carbon monoxide (Kashfi and Olson 2013). Hydrogen sulfide can regulate the central nervous system, with a neuroprotective effect; can

perform homeostatic regulation of blood pressure in the cardiovascular system; can regulate the endocrine system, acting in the pancreatic structure and function; can regulate secretion and motility of the jejunum and the colon from the gastrointestinal system; and can even act in the respiratory system with lung remodeling (di Masi and Ascenzi 2013). Therefore, the use of pharmacological and natural H_2S donating or inhibiting compounds can be useful for therapeutical applications in humans (Olson 2011; Kashfi and Olson 2013).

An advantage of sulfate reducers metabolism is its application for environmental biotechnology. The possibility of sulfate reducers to use a variety of electron donors and acceptors for its metabolism is an advantage when using these microorganisms for the bioremediation of soils, sediments and wastewaters. The principal metabolic end product of sulfate reduction, sulfide, can be used for metal precipitation as metal sulfides that are highly insoluble, allowing the removal of toxic metals including U^{VI}, Cr^{VI}, Tc^{VII}, Rh^{III}, Fe^{III}, Pd^{II}, Mo^{VI} and As^{V} from soils or from industrial and domestic metal contaminated wastewaters (Lloyd *et al.* 2001; Valls and de Lorenzo 2002; Ngwenya and Whiteley 2006; Rashamuse and Whiteley 2007; Barton and Fauque 2009). The metals precipitated can be then recovered and reused making the process profitable. Moreover, SRB inhabit environmental sites contaminated with toxic metals, thus their use for bioremediation is also advantageous, since they developed strategies for resistance to toxic compounds (Bruschi *et al.* 2007). The use of SRB in bioremediation processes has been applied in bioreactors in which the H_2S production is optimized, so that the metal precipitation can be enhanced (Malik 2004). Several

processes have been tested in pilot-scale operations and applied to industries, such as the THIOPAQ® technology, a two stages process for the treatment of contaminated groundwater of the zinc refinery in Budelco, the Netherlands (Hockin and Gadd 2007). In the first stage zinc is precipitated as ZnS in an anaerobic process, and in the second stage excess sulfide is oxidized to elemental sulfur by an aerobic process (Barton and Fauque 2009). Besides metal recovery, elemental sulfur can be removed from the contaminated wastewater system and re-utilized as fertilizer or for the production of sulfuric acid, increasing the economical benefit of this bioprocess (Lens *et al.* 2007).

1.2.4 - SULFATE REDUCTION: THE CENTRAL METABOLIC PATHWAY OF SRB

Sulfur is essential for life, making part of the living matter and composing amino acids like cysteine and methionine. To become biologically useful sulfur has to be in its fully reduced state, but in nature the sulfur biologically available is mostly in an oxidized state, so sulfate has to be reduced to sulfide to become biochemically useful. The assimilatory reduction of sulfate is a biological energy consuming process, in which sulfate is reduced to sulfide, that is then integrated into sulfur-containing organic molecules. This mechanism is performed by bacteria, fungi, algae and plants (Roy and Trudinger 1970). The dissimilatory sulfate reduction pathway is an energy conversion process performed by several Bacteria and a few Archaea, which use this process to produce energy for growth (Postgate 1984).

Dissimilatory sulfate reduction takes place in the cytoplasm were sulfate is activated and reduced by soluble enzymes to sulfite, an intermediary product, and finally sulfite is reduced to sulfide. In total, the process of sulfate reduction to sulfide requires four cytoplasmic enzymes, one ATP and eight electrons for reduction (Muyzer and Stams 2008; Barton and Fauque 2009):

(1.7) $SO_4^{2-} + ATP + 8H^+ + 8\ e^- \rightarrow H_2S + AMP + PP_i$

Sulfate transport across the membrane in SRO is performed by symport with cations, H^+ in freshwater species and Na^+ in salt-dependent or marine species (Cypionka 1987; Kreke and Cypionka 1994). Once inside the cytoplasm sulfate activation is necessary because SO_4^{2-} reduction to SO_3^{2-} is chemically unfavorable [$E^{0'}$ (SO_4^{2-}/SO_3^{2-}) = – 516 mV]. The activation of sulfate to a more favorable compound is performed by ATP sulfurylase or sulfate adenylyltransferase (Sat). The ATP sulfurylase has been purified and characterized from *D. gigas* and *D. desulfuricans* as a novel metalloprotein containing zinc (Gavel *et al.* 1998). Sulfate reacts with ATP at this enzyme to form adenosine-5'-phosphosulfate (APS) and inorganic pyrophosphate (PP_i). The activation product APS has a redox potential much more positive and more favorable for reduction [$E^{0'}$ (APS/SO_3^{2-}) = – 60 mV)]. The reaction is driven by a pyrophosphatase that hydrolyses the inorganic pyrophosphate formed and favors the formation of APS:

(1.8) $SO_4^{2-} + ATP + 2H^+ \rightarrow APS + PP_i$ $\Delta G^{0'} = +46$ kJ/mol

(1.9) $PP_i + H_2O \rightarrow 2P_i \qquad \Delta G^{0'} = -22$ kJ/mol

In *Desulfovibrio* the presence of active soluble pyrophosphatases is well documented (Fauque *et al.* 1991), and membrane associated pyrophosphatases are present in some organisms, Gram-positive SRO, *Caldivirga maquiligensis* and some members of Deltaproteobacteria, which can couple the PP_i hydrolysis to proton translocation across the membrane and contribute to the proton motive force and minimize the energy costs of sulfate reduction (Pereira *et al.* 2011). The activation of sulfate is a common step in the assimilatory and dissimilatory sulfate reduction pathways. APS is the first true electron acceptor in sulfate reduction and APS reduction to sulfite is therefore the first redox reaction:

(1.10) $APS + 2e^- \rightarrow SO_3^{2-} + AMP \qquad E^{0'} = -60$ mV

The enzyme responsible for the reaction, APS reductase (AprBA) has been purified and characterized from several *Desulfovibrio* spp. (Lampreia *et al.* 1994; Lopez-Cortes *et al.* 2005) and from the archaeon *Archaeoglobus fulgidus* (Lampreia *et al.* 1991); it is a non-heme, iron-sulfur protein with a flavin cofactor. The protein arrangement is heterodimeric (αβ) with the α-subunit carrying the FAD cofactor with a molecular mass of 70 – 75 kDa and the β-subunit with two $[4Fe\text{-}4S]^{2+/1+}$ clusters with 18 - 23 kDa (Fritz *et al.* 2000). The first structure of APS reductase was obtained from *A. fulgidus* (Fritz *et al.* 2002b) and later from *D. gigas* (Chiang *et al.* 2009). The mechanism of APS reduction have

been the subject of several studies (Fritz *et al.* 2000; Fritz *et al.* 2002b; Rabus *et al.* 2006; Schiffer *et al.* 2006), and the flavin cofactor in the α-subunit is proposed to be the catalytic site. During the reaction, APS binds to reduced flavin, $FADH_2$, and a sulfonation reaction proceeds by nucleophilic attack of the N5 atom of FAD on the sulfate sulfur of APS. AMP is released and an $FADH_2$-sulfite adduct is formed; following that, the adduct dissociates into oxidized FAD and sulfite. The product sulfite is then released and oxidized FAD is reduced via the iron-sulfur centers of the β-subunit – Figure 1.12 (Rabus *et al.* 2006; Schiffer *et al.* 2006).

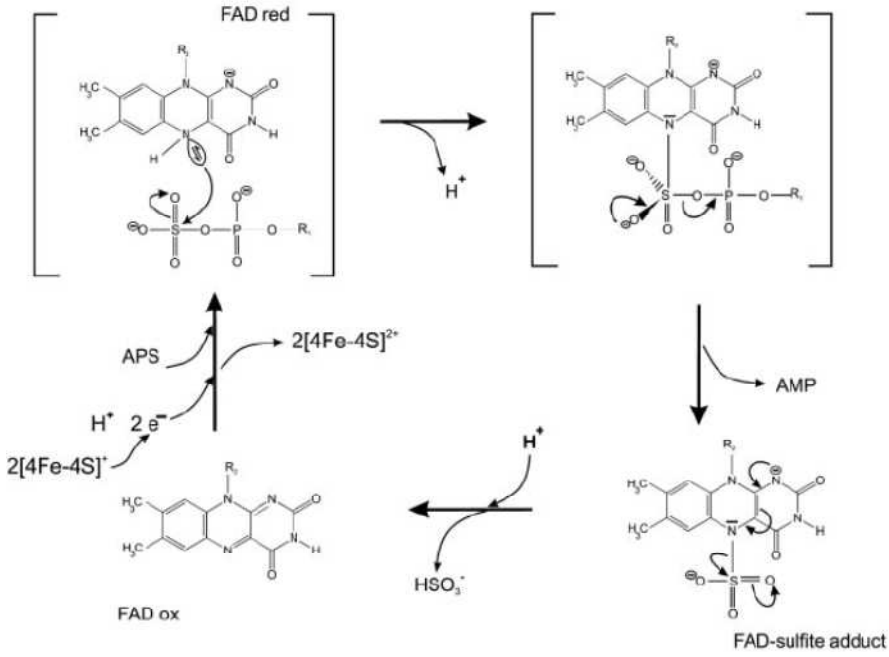


Figure 1.12 - Mechanism of dissimilatory APS reduction to sulfite at the catalytic center FAD adapted from (Fritz *et al.* 2002a). FAD is reduced ($FADH_2$) by the electrons coming from the two [4Fe-4S] clusters. APS binds to $FADH_2$, and nucleophilic attack of N5 results in binding of APS sulfur to reduced FAD. AMP is released and the adduct $FADH_2$-sulfite is formed. The adduct dissociation results in sulfite release and FAD oxidation. R represents the residue that binds the cofactor to the enzyme.

The physiological electron donor to APS reductase is still to be elucidated, but several lines of evidence point to the **Q**uinone-interacting **m**embrane-bound **o**xidoreductase (QmoABC) complex (Pires *et al.* 2003; Haveman *et al.* 2004).The first evidence is that in many organisms, including both SRO and SOB, the *aprBA* genes cluster with the *qmoABC* genes suggesting a possible physiological connection between the two. Additionally, in 2010 Zane and coworkers (Zane *et al.* 2010) proved for the first time that there is a direct link between Qmo and sulfate reduction, since a deletion mutant lacking the *qmoABC* genes was not able to grow on sulfate but grew well in sulfite. Similarly, the sulfur oxidizer *Chlorobaculum tepidum* needs the *qmo* genes to oxidize sulfite (Rodriguez *et al.* 2011). The investigation of a possible interaction between QmoABC and AprBA will be addressed in Chapter 3, section 3.1. The final step in the reduction pathway consists of sulfite reduction to sulfide in a six electron reduction, by the dissimilatory sulfite reductase (dSir or DsrAB):

(1.11) $SO_3^{2-} + 6\ e^- + 8\ H^+ \rightarrow H_2S + 6\ H_2O$ $\qquad E^{0\prime} = -116$ mV

The terminal reductase responsible for the reaction, DsrAB, is present in all SRO investigated so far and contains two metal cofactors, responsible for the electron transfer to the substrate, a siroheme and an iron-sulfur cluster. In SRB, four different types of Dsr's can be distinguished according to UV-visible spectral characteristics and molecular and cofactor content (Rabus *et al.* 2006). The different types of Dsr's are: the green protein desulfoviridin found mainly in *Desulfovibrio* spp. with an

absorption peak at 628 nm (sirohydrochlorin cofactor) (Lee and Peck 1971; Moura *et al.* 1988); the reddish brown protein desulforubidin present in *Desulfomicrobium* and *Desulfosarcina* spp. has characteristic absorption at 545 nm (Lee *et al.* 1973; Moura *et al.* 1988); the dark brown desulfofuscidin, found in *Thermodesulfobacterium* with a characteristic peak at 576 nm (Hatchikian and Zeikus 1983); and the P582, present in *Desulfatomaculum* with an absorption peak at 582 nm (Akagi and Adams 1973). The Dsr's usually have a tetrameric $\alpha_2\beta_2$ arrangement, but a third type of subunit (γ) has been suggested in some *Desulfovibrio* spp. presenting a hexameric structure ($\alpha_2\beta_2\gamma_2$). This third subunit, DsrC, is not encoded in the same operon of DsrA and DsrB, and is in fact not a subunit, but a protein with which DsrAB interacts (Venceslau *et al.* 2013). Structural analysis of DsrAB from *D. vulgaris* showed that this protein forms a tight complex with DsrC, with the DsrC C-terminal arm protruding into a channel formed between DsrA and DsrB (Oliveira *et al.* 2008b).

The last step in the sulfate reduction mechanism has two different pathways proposed: one that considers a sequential reduction in three two-electron steps with the formation of intermediary products (trithionate and thiosulfate) (Kobayashi *et al.* 1974; Akagi 1983); the other, a direct reduction with six electron involved without formation of any intermediates (Steuber *et al.* 1994). It is not clear if trithionate and thiosulfate are intermediary products of the reaction or if they are only side products of the *in vitro* experiments (Chambers and Trudinger 1975; Peck et al 1982; Rabus 2006).

The physiological electron donor of sulfite reductase was proposed to be the membrane complex DsrMKJOP (Mander 2002; Pires 2006), and a four-electron reduction mechanism involving DsrAB, DsrC and DsrMKJOP was proposed (Oliveira *et al.* 2008a). In this new mechanism a sulfur intermediate is formed in the DsrAB active site and is transferred to DsrC to form a persulfide. Once DsrC is released from DsrAB the persulfide is reduced forming sulfide and oxidized DsrC, which in turn can be reduced by DsrMKJOP in a cyclic reaction (Figure 1.13). It is proposed that two electrons come from the quinone pool possibly with proton translocation contributing to the energy conservation of the sulfate reduction. The principal characteristics of DsrMKJOP will be addressed in Chapter 2.

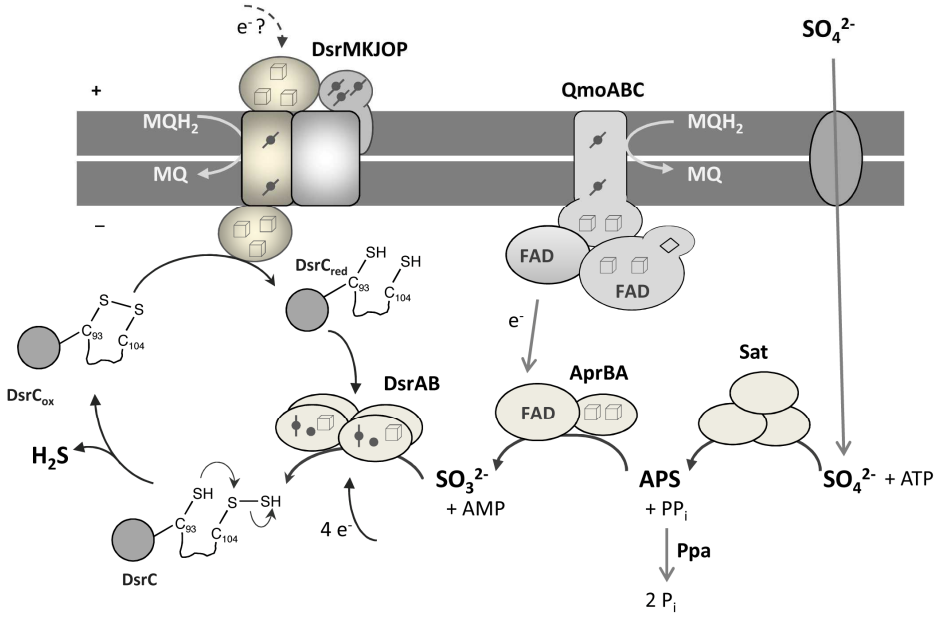


Figure 1.13 - Schematic representation of the sulfate reduction pathway with the involvement of membrane proteins. Sat, sulfate adenylyltransferase; AprBA, APS reductase; QmoABC is the proposed electron donor to APS reductase and DsrMKJOP to DsrC/ DsrAB. Adapted from (Oliveira *et al.* 2008a).

1.2.5 - SULFATE REDUCTION, MEMBRANE PROTEINS AND HDR-LIKE PROTEINS

For many years membrane proteins were not considered to be involved in sulfate reduction, although it was known for a long time that sulfate reduction generates oxidative phosphorylation in a true respiratory process (Peck 1960). In addition, the physiological electron donors to APS reductase and DsrAB were not known and how sulfate reduction is coupled to proton translocation needed to be clarified. These thoughts changed with the isolation and characterization of two conserved membrane proteins present in SRB and also in many SOB: the QmoABC (Pires *et al.* 2003) and the DsrMKJOP complex, first named Hme complex for **H**eterodisulfide reductase-like **m**enaquinol-oxidizing **e**nzyme complex (Mander *et al.* 2002; Pires *et al.* 2006). The Qmo complex was suggested to be involved in electron transfer to APS reductase and the DsrMKJOP complex to DsrAB (Pires *et al.* 2003; Haveman *et al.* 2004; Pires *et al.* 2006). An important characteristic regarding these membrane complexes is that both contain subunits related to subunits of heterodisulfide reductases (Hdr) of methanogens (Figure 1.7 and 1.13).

1.2.5.1 - THE QMOABC COMPLEX

The QmoABC membrane complex is the most probable electron donor to AprBA, in a process that may result in energy conservation. The complex was first isolated and characterized from membranes of *D. desulfuricans* cells by (Pires *et al.* 2003). It is composed of three subunits, one membrane bound (QmoC) and two cytoplasmic (QmoA and QmoB), all of them related to heterodissulfide reductases of methanogens. QmoA is a

cytoplasmic subunit that binds a FAD domain near the N-terminal which is related to a part of HdrA and presents a molecular mass of 48 kDa. QmoB is also a cytoplasmic subunit with 82 kDa molecular mass and is composed of a FAD binding domain (N-terminal), two $[4Fe\text{-}4S]^{2+/1+}$ clusters and in the C-terminal region there is a domain similar to the delta subunit of the F_{420}-non-reducing hydrogenases (MvhD) of methanogens. The N-terminal domain is similar to QmoA and is related to HdrA. QmoC is a unique membrane subunit with 36 kDa containing a hydrophobic domain with six transmembrane helices in the C-terminal which binds two hemes *b*, possibly coordinated by four conserved histidines, and exhibit sequence similarity to HdrE. The hydrophilic domain is located in the cytoplasm and contains two $[4Fe\text{-}4S]^{2+/1+}$ clusters similar to HdrC. The QmoC protein is an interesting fusion protein that belongs to the family of respiratory oxidoreductases, carrying in the same protein the integral membrane subunit with hemes *b* on opposite sides of the membrane, and an electron transfer subunit. The hemes *b* are low spin, with redox potentials of +75 and – 20 mV, and are reduced by menaquinol analogs suggesting that Qmo is envolved in electron transfer from the quinone pool to the cytoplasm coupling sulfate reduction to chemiosmotic energy conservation (Pires *et al.* 2003).

The occurrence of Hdr-related subunits in sulfate reducers supports not only their antiquity together with methanogens and acetogens, but also suggest that there may be alternative mechanisms for energy conservation in sulfate reducers (Pereira *et al.* 2011; Sousa *et al.* 2013).

The presence of soluble HdrA-like subunits points to the existence of flavin-based electron bifurcation mechanisms operating in SRB. In addition, gene expression and sequence analysis identified *hdrABC* genes clustered with a hypothetical hydrogenase in *D. vulgaris* that may be involved in the energy metabolism of sulfate reducers (Haveman *et al.* 2003). Moreover, the presence of Hdr related subunits suggests also that these complexes may also be involved in thiol/disulfide chemistry and/or that thiols may be implicated in the sulfate reduction pathway (Matias *et al.* 2005).

The investigation of the role of the Qmo complex and flavin-based electron bifurcation mechanisms in SRB are the aims of this thesis, and is the principal subjects of Chapters 3 and 4.

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CHAPTER 2

GENOMIC AND EVOLUTIONARY ASPECTS OF DISSIMILATORY SULFATE REDUCTION

CHAPTER 2

SECTION 2.1

A COMPARATIVE GENOMIC ANALYSIS OF ENERGY METABOLISM IN SULFATE REDUCING BACTERIA AND ARCHAEA

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Ana Raquel Ramos performed the genomic analysis of proteins directly involved in the sulfate reduction pathway.

2.1.1 – SUMMARY

The number of sequenced genomes of sulfate-reducing organisms (SRO) has increased significantly in the recent years, providing an opportunity for a broader perspective into this type of energy metabolism. In this work we carried out a comparative survey of energy metabolism genes found in twenty-five available genomes of SRO. This analysis revealed a higher diversity of possible energy conserving pathways than classically considered to be present in these organisms, and permitted the identification of new proteins not known to be present in this group. The *Deltaproteobacteria* (and *Thermodesulfovibrio yellowstonii*) are characterized by a large number of cytochromes c and cytochrome c-associated membrane redox complexes, indicating that periplasmic electron transfer pathways are important in these bacteria. The Archaea and *Clostridia* groups contain practically no cytochromes c or associated membrane complexes. However, despite the absence of a periplasmic space, a few extracytoplasmic membrane redox proteins were detected in the Gram-positive bacteria. Furthermore, we found evidence that cytoplasmic electron bifurcating mechanisms, recently described for other anaerobes, are also likely to play an important role in energy metabolism of SRO. A number of cytoplasmic [NiFe] and [FeFe] hydrogenases, formate dehydrogenases and heterodisulfide reductase-related proteins are likely candidates to be involved in energy coupling through electron bifurcation, from diverse electron donors such as H_2, formate, pyruvate, NAD(P)H, β-oxidation and others. In conclusion, this analysis indicates that energy metabolism of SRO is far more versatile than previously considered, where both chemiosmotic and flavin-based

electron bifurcating mechanisms provide alternative strategies for energy conservation.

2.1.2 - INTRODUCTION

Sulfate-reducing organisms (SRO) are anaerobic prokaryotes found ubiquitously in nature (Rabus *et al.* 2006; Muyzer and Stams 2008). They employ a respiratory mechanism with sulfate as the terminal electron acceptor giving rise to sulfide as the major metabolic end-product. These organisms play an important role in global cycling of sulfur and carbon in anaerobic environments, particularly in marine habitats due to the high sulfate concentration, where they are responsible for up to 50% of carbon remineralization (Jorgensen 1982). Sulfate reduction is a true respiratory process, which leads to oxidative phosphorylation through a still incompletely understood electron-transfer pathway. This electron transport chain links dehydrogenases to the terminal reductases, which are located in the cytoplasm, and therefore, not directly involved in charge translocation across the membrane and generation of transmembrane electrochemical potential. In recent years, the advent of genomic information coupled with biochemical and genetic studies has provided significant advances in our understanding of sulfate respiration, but several important questions remain to be answered including the sites and mechanisms of energy conservation. These studies revealed that sulfate reduction is associated with a set of unique proteins. Some of these proteins are also present in sulfur-oxidizing organisms, whereas others are shared with anaerobes like methanogens. Most biochemical studies have focused on mesophilic *Deltaproteobacterial* sulfate

reducers, mostly *Desulfovibrio* sp. (Matias *et al.* 2005; Rabus *et al.* 2006), but previous analyses indicated that the composition of energy metabolism proteins can vary significantly between different SRO (Rabus *et al.* 2006; Pereira *et al.* 2007a; Junier *et al.* 2010). The increasing number of SRO genomes available from different classes of both Bacteria and Archaea prompted us to perform a comparative analysis of energy metabolism proteins. In this work we report the analysis of twenty five genomes of SRO available at the Integrated Microbial genomes website. This includes three Archaea, seventeen *Deltaproteobacteria* (of the *Desulfovibrionacae, Desulfomicrobiacae, Desulfobacteraceae, Desulfohalobiacae, Desulfobulbaceae* and *Syntrophobacteraceae* family), four *Clostridia* (of the *Peptococcaceae* and *Thermoanaerobacterales* families) and *Thermodesulfovibrio yellowstonii* DSM 11347 of the *Nitrospirae* phylum (Table 2.1). This analysis extends a previous one in which only the *Deltaproteobacteria D. vulgaris* Hildenborough, *D. desulfuricans* G20 (renamed *D. alaskensis* G20 (Hauser *et al.* 2011)) and *Dt. psycrophila* were considered (Pereira *et al.* 2007a).

The work present in this section focuses mainly on genes coding for proteins essential for sulfate reduction and also on conserved membrane redox proteins (QmoABC and DsrMKJOP). We have also analysed genes involved in cytoplasmic electron transfer, especially proteins involved in flavin-based electron bifurcation reactions and heterodisulfide reductase (HDR) like proteins.

2.1.3 - PROTEINS ESSENTIAL FOR SULFATE REDUCTION

As expected, all organisms analyzed contain genes for those proteins long known to be directly involved in sulfate reduction (Rabus *et al.* 2006), including sulfate transporters, ATP sulfurylase (*sat*), APS reductase (*aprBA*) and dissimilatory sulfite reductase (*dsrAB*). The hydrolysis of pyrophosphate is carried out by soluble inorganic pyrophosphatases in most cases, but in a few organisms a membrane-associated proton-translocating pyrophosphatase (Serrano *et al.* 2007) is present, which may allow energy conservation from hydrolysis of pyrophosphate. These include the Gram-positive bacteria (Junier *et al.* 2010), *S. fumaroxidans*, *Dc. oleovorans*, *Df. alkenivorans* and *C. maquilingensis*. F_1F_0-ATP synthases are also present in all the SRO analyzed. Other strictly conserved proteins include ferredoxins, which are very abundant proteins in sulfate reducers (Moura *et al.* 1994). Their crucial role in anaerobic metabolism has gained increasing evidence in recent years (Meuer *et al.* 2002; Herrmann *et al.* 2008; Thauer *et al.* 2008). All organisms analyzed contain ferredoxin I (Fd I), which in some cases is present in multiple copies, and most contain also ferredoxin II (Fd II).

One of the remaining important questions about sulfate reduction is the nature of the electron donors to the terminal reductases AprBA and DsrAB. Two membrane complexes, QmoABC and DsrMKJOP (Figure 2.1; Table 2.1) have been proposed to perform this function (Pereira 2008).

2.1.3.1 - THE QMOABC COMPLEX

QmoABC (for *Q*uinone-interacting *m*embrane-bound *o*xidoreductase complex) was first described in *D. desulfuricans* ATCC 27774 (Pires *et al.* 2003). It includes three subunits binding two hemes *b*, two FAD groups and several iron-sulfur centers. QmoA and QmoB are both soluble proteins homologous to HdrA, a flavin-containing subunit of the soluble HDRs (Hedderich *et al.* 2005). HDRs are key enzymes in methanogens that catalyze the reduction of the CoM-S-S-CoB heterodisulfide, formed in the last step of methanogenesis, to the corresponding thiols (Hedderich *et al.* 2005). The function of HdrA is still not clear, but it has been proposed to be involved in flavin-based electron bifurcation by an HdrABC/MvhADG complex, where the endergonic reduction of ferredoxin by H_2 is coupled to the exergonic reduction of the CoM-S-S-CoB heterodisulfide by H_2 (Thauer *et al.* 2008). QmoC is a fusion protein that contains a cytochrome *b* transmembrane domain related to HdrE and a hydrophilic iron-sulfur domain related to HdrC. QmoB includes also a domain similar to MvhD, a subunit of F_{420}-non-reducing hydrogenase (Mvh) (Thauer *et al.* 2010).

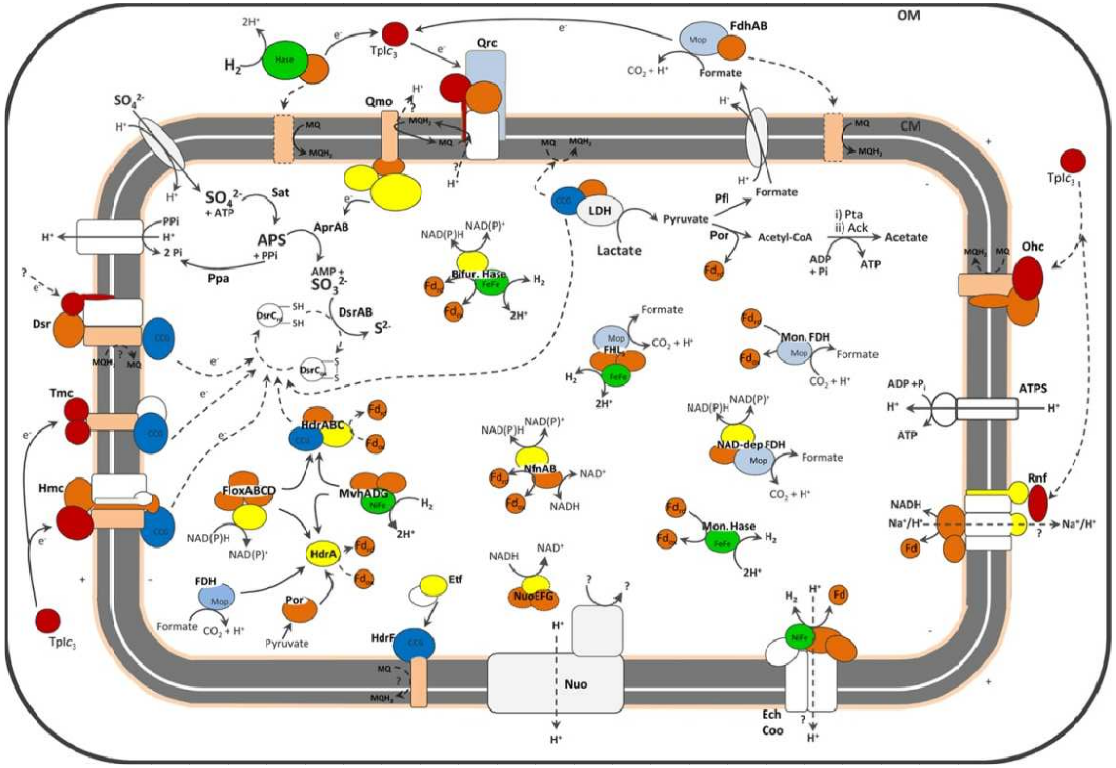

Figure 2.1 – Schematic representation of the cellular location of SRO main energy metabolism proteins. No single organism is represented. The dashed lines represent hypothetical pathways, or (in the case of periplasmic Hases and FDH's) pathways present in only a few organisms. Color code is red for cytochromes c, pale orange for cytochrome b, yellow for flavoproteins, dark orange for FeS proteins, ligh blue for proteins of molybdopterin family, dark blue for CCG proteins and green for catalytic subunits of Hases.

Since the *qmo* genes are usually adjacent to *aprBA*, and both QmoC hemes are reduced by a menaquinol analogue, it has been proposed that Qmo transfers electrons from the quinone pool to AprBA, in a process that may result in energy conservation (Pires *et al.* 2003; Venceslau *et al.* 2010). Although direct electron transfer has not been reported, it was recently shown that in *D. vulgaris* Hildenborough the Qmo complex is essential for sulfate, but not forsulfite, reduction (Zane *et al.* 2010). Our analysis confirmed that a gene locus containing *sat*, *aprBA* and the *qmoABC* genes is present in the majority of SRO analyzed. The exceptions are the archaeon *C. maquilingensis* for which no *qmo* genes are detected, and the Gram-positive bacteria where the *qmoC* gene is absent. In *Desulfotomaculum acetoxidans* and *Candidatus* Desulforudis audaxviator the *qmoC* gene is replaced by the *hdrBC* genes that code for soluble subunits of HDRs (Junier *et al.* 2010). This suggests that in Gram-positive bacteria the reduction of APS reductase may derive from soluble pathways, rather than quinones, and not be coupled to energy conservation.

2.1.3.2 - THE DSRMKJOP COMPLEX

The *dsrMKJOP* genes were first reported in the sulfur-oxidizing bacterium *Allochromatium vinosum* as part of a *dsr* locus encoding also the *dsrAB* and *dsrC* genes, among others (Pott and Dahl 1998). The DsrMKJOP complex was isolated from *Archaeoglobus fulgidus* (Mander *et al.* 2002) (where it was named Hme) and *D. desulfuricans* ATCC 27774 (Pires *et al.* 2006). It is a transmembrane complex with redox subunits in the periplasm - the triheme cytochrome *c* DsrJ, and the iron-sulfur

protein DsrO; in the membrane - the cytochrome *b* DsrM (NarI family), and DsrP (NrfD family); and in the cytoplasm - the iron-sulfur protein DsrK that is homologous to HdrD, the catalytic subunit of the membrane-bound HdrED. DsrK and HdrD are both members of the CCG protein family characterized by a conserved cysteine-rich sequence (CX_nCCGX_mCXXC), which includes over 2000 archaeal and bacterial proteins (Hedderich *et al.* 1999; Hamann *et al.* 2007). This Cys sequence binds a special [4Fe4S] cluster, which in HDR is responsible for heterodisulfide reduction (Hedderich *et al.* 2005), and is also present in Dsr (Pires *et al.* 2006). Sequence analysis suggests that there may be two modules in the Dsr complex. One module, formed by DsrMK (based on its similarity to HdrED), may be involved in menaquinol oxidation and reduction of a cytoplasmic substrate, probably the DsrC disulfide (Oliveira *et al.* 2008); a second module formed by DsrJOP may be involved in electron transfer between the menaquinone pool and a periplasmic component, but it is not clear in which direction. The *dsrMKJOP* genes are present in all SRO genomes analyzed, with the exception of the Gram-positive bacteria (Junier *et al.* 2010) and *C. maquilingensis*, for which only *dsrMK* are present. This indicates that only these two proteins are essential for sulfite reduction. Gram-positive bacteria lack a periplasmic space, which may explain the absence of DsrJO, and in these organisms DsrMK must transfer electrons between the menaquinone pool and the cytoplasm, whereas in organisms with DsrMKJOP electron transfer likely involves also periplasmic components. Several SRO contain both *dsrMKJOP* and one or more copies of *dsrMK*. A

DsrMK protein was isolated from *Archaeoglobus profundus* (Mander *et al.* 2004).

2.1.3.3 – DsrC

The *dsrC* gene is also strictly conserved in all SRO. It is one of the most highly expressed genes in *D. vulgaris* Hildenborough (Haveman *et al.* 2003; Wall *et al.* 2008) and also environmental samples (Canfield *et al.* 2010), pointing to an important role in sulfur metabolism. All organisms encoding a *dsrAB* sulfite reductase (sulfate/sulfite reducers or sulfur oxidizers) also contain the *dsrC* and *dsrMK* genes. DsrC is a small protein with a C-terminal swinging arm containing two strictly conserved cysteines (Cort *et al.* 2001; Mander *et al.* 2005). It belongs to a larger family of proteins, present also in organisms that do not perform dissimilatory sulfur metabolism (*e.g. E. coli* TusE), where they are involved in sulfur-transfer reactions (Ikeuchi *et al.* 2006). In these cases, a single cysteine, the penultimate residue of the C-terminal arm, is conserved. This suggests the involvement of a disulfide bond between the two DsrC cysteines as a redox-active center in the sulfite reduction pathway. DsrC was initially described as a subunit of DsrAB, with which it forms a tight complex (Pierik *et al.* 1992). However, DsrC is not a subunit, but rather a protein with which DsrAB interacts. The crystal structure of the DsrAB-DsrC complex from *D. vulgaris* revealed that the DsrC swinging arm inserts into a cleft between DsrA and DsrB, such that its penultimate cysteine comes in close proximity to the sulfite binding site at the catalytic siroheme (Oliveira *et al.* 2008). A mechanism for sulfite reduction involving DsrC was proposed, in which a DsrC persulfide is

formed and gives origin to oxidised DsrC ($DsrC_{ox}$) with a disulfide bond between the two cysteines (Oliveira *et al.* 2008). $DsrC_{ox}$ is then proposed to be reduced by the DsrK subunit of the Dsr complex, which contains a catalytic iron-sulfur center for putative reduction of disulfide bonds, as described in HDRs (Pires *et al.* 2006). The involvement of the Dsr complex provides a link between membrane quinol oxidation and sulfite reduction that may explain the fact that proton translocation is associated with this reduction (Kobayashi *et al.* 1982). *In vitro* sulfite reduction by desulfoviridin, the dissimilatory sulfite reductase of *Desulfovibrio* spp. does not yield sulfide as observed in the assimilatory enzymes, but a mixture of products including thiosulfate and trithionate (Rabus *et al.* 2006). This led to the proposal that sulfite reduction in SRO proceeds with thiosulfate and trithionate as intermediates (Akagi 1995). In *Desulfovibrio gigas*, flavoredoxin was implicated in thiosulfate reduction (Broco *et al.* 2005). However, flavoredoxin is not conserved across the SRO analyzed and there is also no evidence for enzymes to handle trithionate. Most likely the *in vitro* polythionate products observed originate from the absence of other proteins required for physiological sulfite reduction, namely DsrC (Oliveira *et al.* 2008).

Our genomic analysis of SRO supports the interaction between DsrC, DsrAB and the DsrMKJOP complex: In *A. profundus* and *T. yellowstonii* *dsrC* is in the same gene cluster as *dsrMKJOP*, and in the three Gram-positive organisms and *Ammonifex degensii,* a *dsrMK-dsrC* gene cluster is present (Figure 2.2). Strikingly, this cluster is preceded by a gene encoding a ferredoxin (Fd), and a Fd gene is also present after the *dsrMKJOP* genes and in close proximity to *dsrAB* in three

Deltaproteobacteria. This suggests that a Fd may also be involved in the electron transfer pathway between the Dsr complex, DsrC and DsrAB. The involvement of Fd provides a link between the sulfite reduction step and other soluble electron transfer pathways.

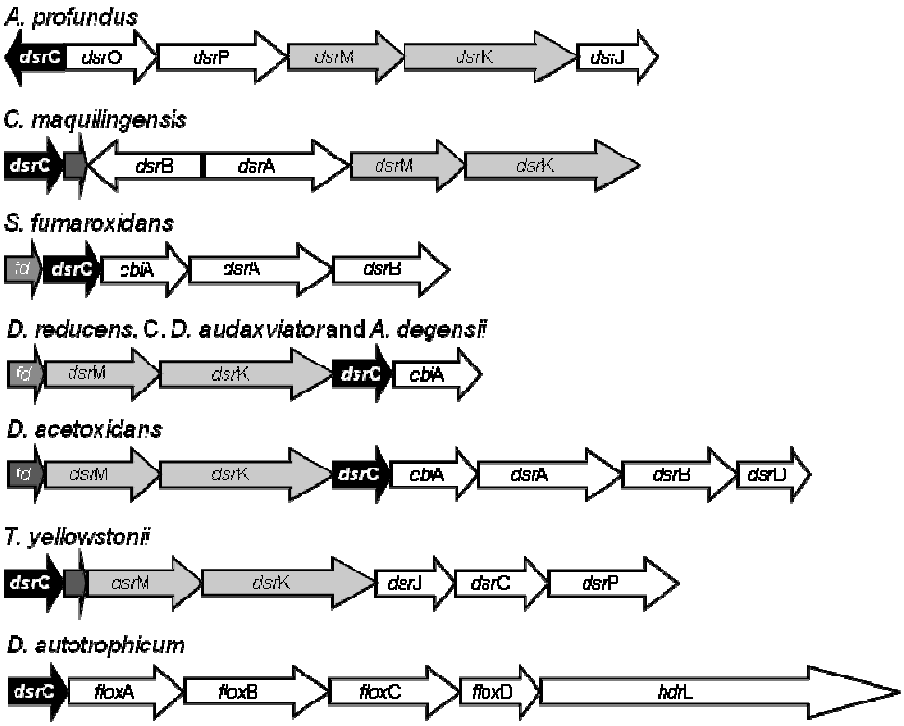

Figure 2.2 - Examples of neighborhood gene organization of the *dsrC* gene; fd, ferredoxin; *cbi*, cobyrinic acid a,c-diamide synthase.

Table 2.1 - Analysis of membrane redox complexes Dsr and Qmo in SRO genomes. MK only *dsrMK* genes present; † only *qmoAB* present.

	Dsr	Qmo	TpIc$_3$	Qrc	Tmc	Hmc	Nhc
Archaea							
Archaeoglobus fulgidus	1 + 2MK	1					
Archaeoglobus profundus	1	1					
Caldivirga maquilingensis	MK						
Deltaproteobacteria							
Desulfovibrionacae							
Desulfovibrio aespoeensis	1	1	2	1	1	1	1
Desulfovibrio desulfuricans G20	1	1	2	1	1	1	
Desulfovibrio desulfuricans ATCC 27774	1	1	1		1		1
Desulfovibrio magneticus RS-1	1	1	2	1	1	1	
Desulfovibrio piger	1	1	1				1
Desulfovibrio salexigens	1	1	3	1	1	1	
Desulfovibrio sp. FW1012B	1	1	2	1	1	1	
Desulfovibrio vulgaris Hildenborough	1	1	1	1	1	1	
Desulfomicrobiacae							
Desulfomicrobium baculatum	1	1	2	1	1	1	
Desulfobacteraceae							
Desulfatibacillum alkenivorans	1	1	2	1			1
Desulfobacterium autotrophicum HRM2	1	1	1	2	2		
Desulfococcus oleovorans Hxd3	1	1	2	1	2	1	
Desulfohalobiacae							
Desulfohalobium retbaense DSM 5692	1	1	4	1	1	1	
Desulfonatronospira thiodismutans ASO3-1	1	1	3		2		1
Desulfobulbaceae							
Desulfotalea psychrophila	1	1					
Desulfurivibrio alkaliphilus	1	1	2				
Syntrophobacteraceae							
Syntrophobacter fumaroxidans MPOB	1	1	1	1			
Clostridia							
Peptococcaceae							
Desulfotomaculum acetoxidans DSM 771	MK	1†					
Desulfotomaculum reducens	MK	1†					
C. Desulforudis audaxviator MP104C	MK	1†					
Thermoanaerobacterales							
Ammonifex degensii KC4	MK	1					
Nitrospirae							
Thermodesulfovibrio yellowstonii	1	1	1			1	
No. of organisms	20/5	24	17	12	12	10	5

2.1.4 - CYTOPLASMIC ELECTRON TRANSFER

In recent years several studies unraveled a novel process of coupling endergonic to exergonic redox reactions in anaerobic organisms, through a flavin-based electron bifurcation mechanism involving only soluble proteins (Herrmann *et al.* 2008; Li *et al.* 2008; Thauer *et al.* 2008; Schut and Adams 2009). This mechanism involves the two-step reduction/oxidation of a flavin cofactor, through a flavin-semiquinone intermediate, where each step is associated with a different reductant/oxidant (Thauer *et al.* 2008), in analogy to the quinone-based electron bifurcating mechanism of the bc_1 complex (Xia *et al.* 2007). Five examples have been described including: i) the coupling of ferredoxin (Fd) reduction with NADH to reduction of butyryl-CoA with NADH by the butyryl-CoA dehydrogenase-EtfAB complex (Herrmann *et al.* 2008; Li *et al.* 2008), ii) coupling of Fd reduction with H_2 to the reduction of the methanogenic CoM-S-S-CoB heterodisulfide with H_2 catalyzed by the MvhADG/HdrABC complex (Thauer *et al.* 2008; Thauer *et al.* 2010), iii) coupling of Fd reduction with formate to the reduction of the methanogenic CoM-S-S-CoB heterodisulfide with formate catalyzed by a FdhAB/HdrABC complex (Costa *et al.* 2010), iv) coupling of H_2 formation from NADH with H_2 formation from reduced Fd catalyzed by the multimeric [FeFe] Hases (Schut and Adams 2009) and v) coupling of $NADP^+$ reduction with reduced Fd with $NADP^+$ reduction with NADH catalyzed by NfnAB (Wang *et al.* 2010). These cases stress the important role Fd plays in anaerobic metabolism. The reduced Fd produced through a bifurcating reaction may be oxidized by membrane-associated ion translocating complexes (such as Rnf or Ech), resulting in energy

conservation, or it may be used as electron donor in other metabolic reactions. Our genomic analysis of SRO revealed there are several examples of soluble proteins in these organisms with the potential to carry out electron bifurcation, from H_2, formate or other carbon-based electron donors. In particular, a very high number of proteins related to HDRs were identified.

2.1.4.1 - CYTOPLASMIC HASES

An unexpected high number of soluble cytoplasmic hydrogenases, of both [NiFe] and [FeFe] families, were detected in the present analysis (Table 2.2). Most organisms contain a cytoplasmic-facing Hase, either soluble or membrane-bound, except the two organisms that contain no Hases at all and *Desulfomicrobium baculatum*. In numerous cases, the gene organization indicates that the cytoplasmic Hases are likely to be involved in electron bifurcation mechanisms. A large number of the [NiFe] Hases detected are related to the MvhADG Hases of methanogens (Thauer *et al.* 2010). In these organisms MvhADG reduces the cytoplasmic heterodisulfide reductase HdrABC, and the two proteins have been shown to form a large complex (Stojanowic *et al.* 2003). The activity of this complex is increased in the presence of Fd, and MvhADG/HdrABC are proposed to couple the endergonic reduction of Fd with H_2 to the exergonic reduction of the heterodisulfide with H_2 by electron bifurcation, probably involving the FAD group of HdrA (Thauer *et al.* 2008; Thauer *et al.* 2010). In the SRO analyzed the *mvhADG* genes are found next to an *hdrA* gene (6 organisms) or *hdrABC* genes (4 organisms), suggesting these act as electron acceptors in a process that

may involve electron bifurcation. In five organisms no *hdr* genes are close by. Another type of closely related [NiFe] Hase, of the Hox type, is present only in three organisms. Hox Hases are bidirectional NADP-linked Hases common in cyanobacteria, and also found in other organisms (Vignais and Billoud 2007). In the three SRO the Hox gene cluster includes *hoxHY* coding for the catalytic and small subunits, and *hoxEFG* that are homologous to *nuoEFG*, and code for the diaphorase module of the Hase. It is striking that in all SRO analyzed, with a single exception (*C.* Dr. audaxviator), the organisms that contain the energy-conserving Hases Ech or Coo do not contain other cytoplasmic [NiFe] Hases, and the same is true *vice-versa*. This suggests that in SRO energy coupling through [NiFe] Hases involves either a chemiosmotic or an electron-bifurcating mechanism. In the Archaea only MvhADG/HdrABC is detected, and in the *Clostridia* only two isolated MvhADG Hases are present. In two organisms, genes for another [NiFe] Hase are found next to genes encoding sensor/response-regulator proteins and histidine kinases, suggesting they are regulatory Hases.

Many cytoplasmic [FeFe] Hases are also present in the SRO analyzed, and are particularly abundant in the *Clostridia* class. Many of these are monomeric Fd-dependent Hases (Table 2.2). Another large group of [FeFe] Hases detected is formed by multimeric NAD(P)-dependent Hases similar to the tetrameric Hases from *D. fructosovorans* (Malki *et al.* 1995) and *Thermoanaerobacter tengcongensis* (Soboh *et al.* 2004). These enzymes include one flavoprotein subunit that binds NAD(P). Another member of this group is the trimeric Hase of *Thermotoga maritima* that was shown to use Fd and NADH synergistically as electron donors for

production of H_2 (Schut and Adams 2009). This is proposed to be also an electron bifurcating mechanism in which the exergonic oxidation of Fd is coupled to the unfavorable oxidation of NADH to give H_2. In *D. fructosovorans* cell extracts no NAD^+-reducing activity was detected and it was proposed that the enzyme functions as a $NADP^+$-reducing H_2-uptake Hase (Malki *et al.* 1995). The enzyme from *T. tengcongensis* was isolated and shown to work bidirectionally with NAD(H), but not with NADP(H) (Soboh *et al.* 2004). In the organisms analyzed the enzyme may be tetrameric, trimeric and in two organisms (*D. vulgaris* and *Db. autotrophicum*) dimeric. At this point it is not clear if the function of these Hases in the SRO is of H_2 production from Fd/NAD(P)H, the reverse, or both depending on the metabolic conditions.

A novel and interesting group of [FeFe] Hases genes are found next to a gene coding for a type I formate dehydrogenase (FDH) (Matson *et al.* 2010), suggesting the two units may form a soluble formate-hydrogen lyase complex (FHL_s). This gene cluster is present only in 5 *Deltaproteobacteria*, and includes minimally the gene coding for the iron-only Hase, the gene for the catalytic subunit of FDH and two four-cluster electron transfer proteins related to HydN. All subunits are soluble in contrast to the *E. coli* FHL complex (Sawers 2005). In some organisms, the iron-sulfur protein encoded next to the *hydA* gene has a predicted signal peptide, but this is absent in other organisms. This raises doubts about the cellular location of the Hase. It is possible that this sequence is not cleaved and acts as a membrane anchor. This putative FHL_s complex is equivalent of the one recently described to be present in the termite gut acetogen *Treponema primitia*, where it is proposed to

carry out H_2-dependent CO_2 reduction (Matson *et al.* 2010). However, the function of these proteins in SRO remains for now unknown.

Finally, in six organisms an [FeFe] Hase including a PAS sensor domain was identified, which is very similar to the HsfB protein recently reported in *Thermoanaerobacterium saccharolyticum* (Shaw *et al.* 2009). This Hase is likely to be involved in H_2 sensing and regulation.

2.1.4.2 - ELECTRON BIFURCATING TRANSHYDROGENASE

A heterodimeric transhydrogenase was recently reported from the *Clostridium kluyveri* (Wang *et al.* 2010). The enzyme, named NfnAB, catalyzes the reversible NADH-dependent reduction of $NADP^+$ by reduced Fd, or the NAD^+-dependent reduction of Fd by NADPH. It is another example of a bifurcating reaction as it couples the exergonic reduction of $NADP^+$ with reduced Fd to the endergonic reduction of $NADP^+$ with NADH. The *nfnAB* genes, both encoding iron-sulfur flavoproteins, are present in several organisms (Wang *et al.* 2010). They are often annotated as sulfide dehydrogenase, as this enzyme was initially reported in *Pyrococcus furiosus* to act as sulfide dehydrogenase (Ma and Adams 1994), but later described to act physiologically as a Fd:$NADP^+$ oxidoreductase (Ma and Adams 2001).

Table 2.2 - Analysis of cytoplamsic Hase distribution in the SRO genomes. N_T, total number of Hases; $[FeFe]_{bif}$, cytoplasmic NAD(P)-dependent Hases; $[FeFe]_{mon}$, monomeric Fd-dependent Hases.

		Cytoplasmic [NiFe]							Cytoplasmic [FeFe]			
		Soluble					Memb		Soluble			
	N_T	HdrA-Mvh	HdrABC-Mvh	Mvh	Hox	Sensor	Ech	Coo	[FeFe] bif	[FeFe] mon	FHL	HsfB
Archaea												
Archaeoglobus fulgidus	1		1									
Archaeoglobus profundus	1		1									
Caldivirga maquilingensis												
Deltaproteobacteria												
Desulfovibrionacae												
Desulfovibrio aespoeensis	1						1					
Desulfovibrio desulfuricans G20	2									1	1	
Desulfovibrio desulfuricans ATCC 27774	2						1	1				
Desulfovibrio magneticus RS-1	5						1		2		1	1
Desulfovibrio piger	2							1		1		
Desulfovibrio salexigens	2						1				1	
Desulfovibrio sp. FW1012B	3						1		1			1
Desulfovibrio vulgaris Hildenborough	3						1	1	1			
Desulfomicrobiacae												
Desulfomicrobium baculatum	0											
Desulfobacteraceae												
Desulfatibacillum alkenivorans	2	1				1						
Desulfobacterium autotrophicum HRM2	4	1							1		1	1
Desulfococcus oleovorans Hxd3												
Desulfohalobiacae												
Desulfohalobium retbaense DSM 5692	1	1										
Desulfonatronospira thiodismutans ASO3-1	3	1	1			1						
Desulfobulbaceae												
Desulfotalea psychrophila	4	1			1					1	1	
Desulfurivibrio alkaliphilus	2			1	1							
Syntrophobacteraceae												
Syntrophobacter fumaroxidans MPOB	7	2		1	1				1	1		1
Clostridia												
Peptococcaceae												
Desulfotomaculum acetoxidans DSM 771	3								1	1		1
Desulfotomaculum reducens	6								3	2		1
C. Desulforudis audaxviator MP104C	6			1			1		1	3		
Thermoanaerobacterales												
Ammonifex degensii KC4	3			1						2		
Nitrospirae												
Thermodesulfovibrio yellowstonii	4		1	1						2		
Nº of organisms		7	4	5	3	2	7	3	8	9	5	6

We found that the *nfnAB* genes are also present in the great majority of SRO, with the exception of the Archaea, and three bacteria (Supplementary material), suggesting it plays an important role also in the metabolism of SRO.

2.1.4.3 - HETERODISULFIDE REDUCTASE-LIKE PROTEINS

In methanogens without cytochromes the HDR enzyme is soluble and composed of three subunits, HdrABC, whereas in methanogens with cytochromes it is membrane-associated and formed by two subunits, HdrDE (Hedderich *et al.* 2005; Deppenmeier and Müller 2008; Thauer *et al.* 2008). HdrA is an iron-sulfur flavoprotein, HdrC is a small iron-sulfur protein and HdrB contains two CCG domains and harbors a special [4Fe4S] catalytic site. HdrE is a membrane-bound cytochrome *b* and HdrD has both HdrB- and HdrC-like domains and includes a similar catalytic cofactor to HdrB. The HdrDE protein receives electrons from methanophenazine and reduction of the heterodisulfide is coupled to energy conservation by a redox loop mechanism involving also the membrane-associated VhoACG Hase (Hedderich *et al.* 2005; Deppenmeier and Müller 2008; Thauer *et al.* 2008). The soluble HdrABC enzyme forms a complex with the soluble MvhADG Hase that catalyses heterodisulfide reduction with H_2. This exergonic reaction is proposed to be coupled to the endergonic reduction of Fd by flavin-based electron bifurcation involving HdrA (Thauer *et al.* 2008). As discussed above, the membrane complexes Qmo and Dsr include subunits related to HDRs (Pereira 2008). The abundance of HDR-like proteins in SRO has been highlighted in recent genomes of SRO (Strittmatter *et al.* 2009; Junier *et*

al. 2010). Recently, (Strittmatter *et al.* 2009) proposed two new types of HDR subunits, based on proteins encoded in the *Db. autotophicum* genome. The first, HdrF includes HdrB- and HdrC-like domains fused to a third transmembrane domain. Thus, HdrF is like a fusion of HdrE and HdrD. The second, HdrL, is a large protein containing an HdrA domain and one or two NADH-binding domains (Strittmatter *et al.* 2009). We have analyzed genes coding for HdrA-, HdrB- and HdrD-like proteins as these are the most relevant subunits of HDRs. In general, we found few HdrB-like proteins and they are either associated with HdrAs or they are domains of HdrDs. In contrast, we found a very high number of HdrA- and HdrD- related proteins in the genomes of SRO. We focus our analysis on HdrA-related proteins (Table 2.3, Section 2.1.4.3.1).

2.1.4.3.1 – HDRA

The majority of HdrA-like proteins are encoded in two types of gene loci (Figure 2.3, Table 2.3). In the first type an *hdrA* gene or a set of *hdrABC* genes are found next to *mvhDGA* genes coding for a soluble Mvh [NiFe] Hase as discussed above. In the second type, again a single *hdrA* gene or a set of *hdrABC* genes are found next to four genes that we named *floxABCD* genes (for *fl*avin *ox*idoreductase). The *floxABCD/hdrABC* gene cluster was first identified in *D. vulgaris* Hildenborough as encoding a putative Hase-HDR complex (Haveman *et al.* 2003), as the *flox* genes are annotated as putative Hase genes because they code for proteins related to subunits of *P. furiosus* NAD(P)-dependent soluble Hases (SH) I and II (Jenney and Adams 2008). However, a gene coding for a catalytic Hase subunit is not present, so Flox is not a Hase. The *floxA* gene codes for a

protein with both FAD and NAD(P)-binding domains and is similar to *P. furiosus* SH subunit γ. The *floxB* and *floxC* genes are related to *rnfC* and both code for iron-sulfur proteins similar to *P. furiosus* SH subunit β. The *floxD* gene codes for a protein similar to MvhD, which in methanogens is involved in electron transfer from Mvh Hase to Hdr (Stojanowic *et al.* 2003). In several organisms the *floxCD* genes are fused into a single gene. Thus, the Flox proteins are likely to oxidize NAD(P)H and transfer electrons to the HdrABC proteins. In *D. vulgaris* and other organisms the *floxABCD/hdrABC* genes are found next to a co-regulated *adh* gene coding for an alcohol dehydrogenase (Haveman *et al.* 2003). The Adh may reduce NAD^+ to NADH, which will be oxidized by Flox. The *floxABCD/hdrA* or *floxABCD/hdrABC* genes are present in the majority of the SRO analyzed. This suggests they play an important physiological role, and indeed these genes have been reported in several gene expression and proteomic studies of *D. vulgaris* energy metabolism (Haveman *et al.* 2003; Zhang *et al.* 2006a; Zhang *et al.* 2006b; Caffrey *et al.* 2007; Pereira 2008; Walker *et al.* 2009). The HdrA-associated Mvh and Flox proteins probably constitute parallel pathways for HdrA reduction from H_2 or NAD(P)H. It seems likely that these proteins may be involved in electron bifurcating reactions involving HdrA as previously suggested (Thauer *et al.* 2008). We further propose that the electron acceptor of the HdrBC proteins may be $DsrC_{ox}$, also thought to be a substrate for DsrK (Oliveira *et al.* 2008). Thus, in SRO the HdrABC/MvhDGA and HdrABC/FloxABCD complexes may provide a soluble route of electron transfer to sulfite reduction through DsrC, where energy coupling occurs through electron

Table 2.3 - Analysis of HdrA-like proteins in the SRO genomes.

	Hdr/Mvh		Hdr/Flox		HdrA/ other		
	HdrABC/ Mvh	**HdrA/ Mvh**	**HdrABC/ Flox**	**HdrA/ Flox**	**HdrAL /Fdh**	**HdrA/ Fdh**	**HdrAL /POR**
Archaea							
Archaeoglobus fulgidus	1						
Archaeoglobus profundus	1						
Caldivirga maquilingensis							
Deltaproteobacteria							
Desulfovibrionacae							
Desulfovibrio aespoeensis					1		
Desulfovibrio desulfuricans G20			2				
Desulfovibrio desulfuricans ATCC 27774			1				
Desulfovibrio magneticus RS-1			1				
Desulfovibrio piger			1				
Desulfovibrio salexigens			1				
Desulfovibrio sp. FW1012B			1				
Desulfovibrio vulgaris Hildenborough			1				
Desulfomicrobiacae							
Desulfomicrobium baculatum			1		1		
Desulfobacteraceae							
Desulfatibacillum alkenivorans		1		1			1
Desulfobacterium autotrophicum HRM2		1		1	1	3	
Desulfococcus oleovorans Hxd3							
Desulfohalobiacae							
Desulfohalobium retbaense DSM 5692		1	2		1		
Desulfonatronospira thiodismutans ASO3-1	1	1	1				
Desulfobulbaceae							
Desulfotalea psychrophila		1					
Desulfurivibrio alkaliphilus						1	
Syntrophobacteraceae							
Syntrophobacter fumaroxidans MPOB		2		2	1		1
Clostridia							
Peptococcaceae							
Desulfotomaculum acetoxidans DSM 771			2	1	2		
Desulfotomaculum reducens				1	1		
C. Desulforudis audaxviator MP104C			1				
Thermoanaerobacterales							
Ammonifex degensii KC4			1				
Nitrospirae							
Thermodesulfovibrio yellowstonii	1						
Nº of organisms	4	6	13	5	7	2	2

bifurcation rather than chemiosmotically through DsrMK. In support of this hypothesis the *dsrC* gene of *Db. autotrophicum* is found next to a *hdrA(L)/floxACBD* gene cluster.

Other gene loci in SRO containing *hdrA*-like genes include a *fdhA* gene (and an *hdrL*) or genes for a pyruvate:Fd oxidoreductase (Por), suggesting that formate and pyruvate may also be the source of electrons for HdrA reduction.

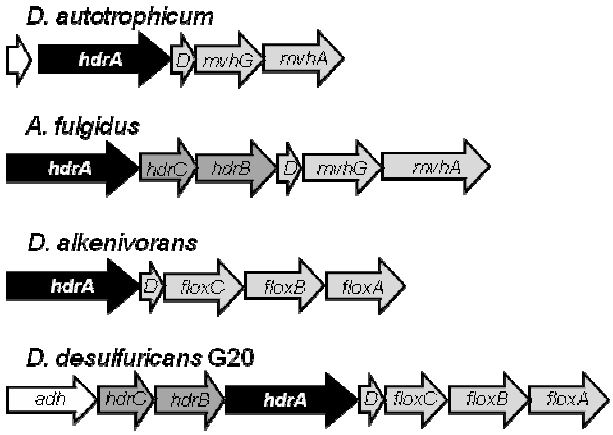

Figure 2. 3 - Examples of gene loci for *hdrA*-related genes (in white lettering).

2.1.5 - CONCLUDING REMARKS

The comparative genomic analysis reported in this work provides new insights into the energy metabolism of SRO. By comparing phylogenetically distinct organisms capable of sulfate reduction we identified the proteins that can be considered as comprising the minimal set required for this metabolic activity: a sulfate transporter, Sat, a pyrophosphatase, AprBA, DsrAB, DsrC, DsrMK and Fd. The QmoAB

proteins are also present in most organisms, being absent only in *C. maquiligensis*. In addition, we identified a higher diversity of possible energy conserving pathways than classically considered to be present in these organisms.

Overall, this analysis suggests that all SRO use diverse processes for energy conservation involving membrane-based chemiosmotic mechanisms, or soluble flavin-based electron bifurcation ones. We identified a surprisingly high number of cytoplasmic Hases and FDHs as likely candidates for electron bifurcation coupling involving NAD(P)/H, Fd or HDRs. A high number of HDR-related proteins were also detected. We propose that these proteins are part of electron transfer pathways involving energy coupling through electron bifurcation, from diverse electron donors such as H_2, formate, pyruvate, NAD(P)H, β-oxidation and others. These pathways may constitute alternatives to Dsr and other transmembrane complexes for reduction of $DsrC_{ox}$, the protein we propose is central to the sulfite reduction step.

A few novel redox proteins were identified in SRO, including a FloxABCD/HdrA(BC) complex proposed to perform electron bifurcation with NAD(P)H, Fd and $DsrC_{ox}$, a new type of membrane-anchored periplasmic [FeFe] Hase, and a putative soluble FHL also comprising an [FeFe] Hase. In conclusion, this analysis indicates that energy metabolism of SRO is far more versatile than previously considered, where both chemiosmotic and flavin-based electron bifurcating mechanisms provide alternative strategies for energy conservation. An interesting aspect of (at least some) SRO is their ability to grow syntrophically in the absence of sulphate. In such situation some modules of this versatile redox

machinery may operate in the opposite direction to that of respiratory conditions. Finally, it should be stressed that although drawing theories based on comparative genomic analysis is an attractive and even convincing exercise, no definite conclusions can be drawn until experimental evidence is provided. Thus, much work remains to be carried out to elucidate the bioenergetic mechanisms of SRO.

2.1.6 - ACKNOWLEDGMENTS

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2.1.7 – SUPPLEMENTARY MATERIAL

The loci for all genes discussed can be found in Appendix I.

CHAPTER 2

SECTION 2.2

UNIFYING CONCEPTS IN ANAEROBIC RESPIRATION: INSIGHTS FROM DISSIMILATORY SULFUR METABOLISM

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Ana Raquel Ramos participated in writing this review.

2.2.1 - SUMMARY

Behind the versatile nature of prokaryotic energy metabolism is a set of redox proteins having a highly modular character. It has become increasingly recognized that a limited number of redox modules or building blocks appear grouped in different arrangements, giving rise to different proteins and functionalities. This modularity most likely reveals a common and ancient origin for these redox modules, and is obviously reflected in similar energy conservation mechanisms.

The dissimilation of sulfur compounds was probably one of the earliest biological strategies used by primitive organisms to obtain energy. Here, we review some of the redox proteins involved in dissimilatory sulfur metabolism, focusing on sulfate reducing organisms, and highlight links between these proteins and others involved in different processes of anaerobic respiration. Noteworthy, are links to the Complex Iron-Sulfur Molybdoenzyme family, and heterodisulfide reductases of methanogenic archaea. We discuss how chemiosmotic and electron bifurcation/confurcation may be involved in energy conservation during sulfate reduction, and how introduction of an additional module, multiheme cytochromes *c*, opens an alternative bioenergetic strategy that seems to increase metabolic versatility.

2.2.2– INTRODUCTION

The dissimilatory metabolism of sulfur compounds is likely to have been among the earliest energy-yielding processes to sustain life with records of more than 3.5 billion years ago (Canfield and Raiswell 1999; Tice and Lowe 2004; Canfield *et al.* 2006; Hohmann-Marriott and Blankenship

2011). It is now clear that there was an intimate connection between the history of Earth's atmosphere and the biogeochemical cycle of sulfur (reviewed in (Farquhar *et al.* 2010; Lyons and Gill 2010)), especially the rise of oxygen in the atmosphere (~2.45 billion years ago) that promoted the increase of the oceanic sulfate concentration from weathering of sulfide minerals on land (Canfield 1998; Canfield *et al.* 2000; Habicht *et al.* 2002; Farquhar *et al.* 2007). It was in the Neoproterozoic Era that deep ocean waters became oxygenated and the sulfate levels rose to present day levels (28 mM), marking the start of the modern sulfur cycle, where biological sulfate reduction plays a major role, particularly in marine sediments where it is responsible for about 50% of carbon remineralization (Jorgensen 1982).

The start of widespread biological sulfate reduction between 2.45 and 2.35 billion years ago is derived from the large increase in mass-dependent sulfur isotope fractionations observed during this period (reviewed in (Farquhar *et al.* 2010; Johnston 2011)). A limited incidence of biological sulfate reduction in the very early Earth is also reflected in the fact that this metabolic trait is not dispersed among prokaryotic organisms, and might have initially been restricted to some early branching thermophilic sulfate reducers. The emergence of mesophilic sulfate reducing organisms (SRO) apparently coincided, or shortly followed the increase in oceanic sulfate levels (Blank 2004; Blank 2009). This radiation of mesophilic SRO seems to have taken place after the rapid diversification of bacterial lineages observed during the Archaean eon, where a significant expansion of energy metabolism genes apparently occurred (David and Alm 2011).

A striking feature of energy metabolism/respiratory proteins is their modular character, which has been described as being based on a "redox protein construction kit" (Baymann *et al.* 2003), from which different combinations of a limited number of protein modules originates different protein complexes with diverse physiological functions. This modular character, which is observed in many protein families, denotes a conservative approach from Nature in using a limited number of original parts to derive new metabolic features. However, it probably also reflects the high level of gene exchange that was present in the pool of LUCA organisms (Woese 2000), as well as the high incidence of lateral gene transfer in later prokaryotes (Boucher *et al.* 2003). In sulfur-metabolizing organisms we find interesting and unique variations of respiratory proteins that reflect their ancient origin and their close environmental association with other anaerobic organisms, in particular with methanogens. In this section we will focus on evolutionary aspects of respiratory proteins involved in dissimilatory sulfur metabolism, focusing on SRO, and discuss new "parts" of the "redox protein construction kit" that are strongly associated with sulfur metabolism but show also links to other respiratory proteins (Figure 2.4) (Pereira *et al.* 2011).

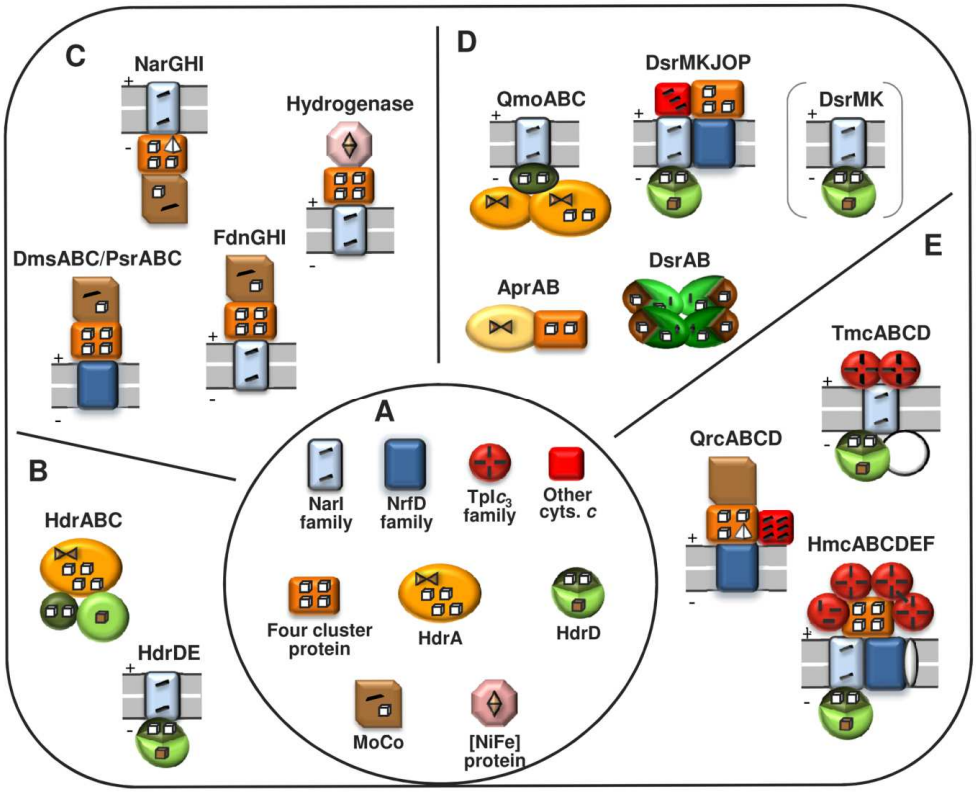

Figure 2.4 - Modular nature of sulfate respiration and related proteins. (A) Redox modules i.e. building blocks from the "redox construction kit" (Baymann *et al.* 2003) that pertain to SRO. (B) Heterodisulfide reductases of methanogens. (C) Trimeric respiratory enzymes including the CISM family and others (Hyn hydrogenase). (D) Conserved respiratory proteins of SRO. (E) Periplasmic and membrane complexes of cytochrome-rich SRO (mainly Deltaproteobacteria). The proteins and respective cofactors are represented schematically (see text descriptions).

2.2.3- The AprBA terminal reductase and its evolution

There are two biological pathways of sulfate reduction. In the assimilatory pathway, which is widespread in the three domains of life, sulfate is reduced to sulfide in small amounts and this is transformed into cysteine, from which other biological sulfur-containing molecules

are derived (Leustek *et al.* 2000). In the dissimilatory pathway, which is restricted to five bacterial and two archaeal lineages, sulfate is the terminal electron acceptor of the respiratory pathway producing large quantities of sulfide (Rabus *et al.* 2006; Muyzer and Stams 2008; Barton and Fauque 2009). The two pathways (Figure 2.5) start with activation of sulfate by reaction with ATP to form adenosine-5´-phosphosulfate (APS), a step catalyzed by the trimeric sulfate adenylyl transferase (Sat), also known as ATP sulfurylase (Ullrich *et al.* 2001; Taguchi *et al.* 2004). The formation of APS is endergonic and is driven by hydrolysis of the pyrophosphate formed by a pyrophosphatase (soluble or membrane-bound). So, the activation of sulfate to APS is considered to consume two ATP equivalents. In the prokaryotic assimilatory pathway APS is converted to 3'-phosphoadenosine-5´-phosphosulfate (PAPS) by the adenylyl sulfate kinase (CysC), PAPS is reduced to sulfite by a thioredoxin-dependent PAPS reductase (CysH), and finally sulfite is reduced to sulfide by an assimilatory sulfite reductase that is either multimeric and NADPH-dependent (CysIJ) or a monomeric ferredoxin-dependent enzyme (Crane and Getzoff 1996). In the dissimilatory pathway APS is reduced to sulfite by the APS reductase (AprBA), a heterodimeric iron-sulfur flavoenzyme (Lampreia *et al.* 1994; Speich *et al.* 1994; Fritz *et al.* 2002a; Fritz *et al.* 2002b). Sulfite is reduced by the dissimilatory sulfite reductase DsrAB, a siroheme containing protein (Moura *et al.* 1988; Dahl *et al.* 1993), with the involvement of the small protein DsrC (Cort *et al.* 2001; Dahl *et al.* 2005; Mander *et al.* 2005; Pires *et al.* 2006; Oliveira *et al.* 2008). Another small protein DsrD, which is often encoded downstream of *dsrAB*, might also be involved in sulfite

reduction, possibly in a regulatory role, but its exact function is still unknown (Mizuno *et al.* 2003). Interestingly, the *dsrD* gene is strongly downregulated in the presence of high sulfide concentrations (Caffrey and Voordouw 2010).

In many anoxygenic phototrophic and chemolithotrophic sulfur oxidizing bacteria (Soboh *et al.*), the Sat, AprBA, DsrAB and DsrC proteins are also present, and thought to be involved in reverse oxidative reactions (reviewed in (Frigaard and Dahl 2009)). DsrAB and DsrC (and the associated DsrMKJOP complex) are also present in organisms that reduce sulfite, thiosulfate or organosulfonate compounds.

The evolution of the dissimilatory sulfate reduction pathway has been investigated by phylogenetic analysis of the *sat* (Sperling *et al.* 1998; Boucher *et al.* 2003), *aprBA* (Hipp *et al.* 1997; Friedrich 2002; Boucher *et al.* 2003; Meyer and Kuever 2007a; Meyer and Kuever 2007b) and mostly of the *dsrAB* genes (Hipp *et al.* 1997; Molitor *et al.* 1998; Wagner *et al.* 1998; Larsen *et al.* 1999; Klein *et al.* 2001; Boucher *et al.* 2003; Loy *et al.* 2008; Loy *et al.* 2009). These studies indicate a mostly vertical inheritance for these genes, but also several episodes of lateral gene transfer (LGT).

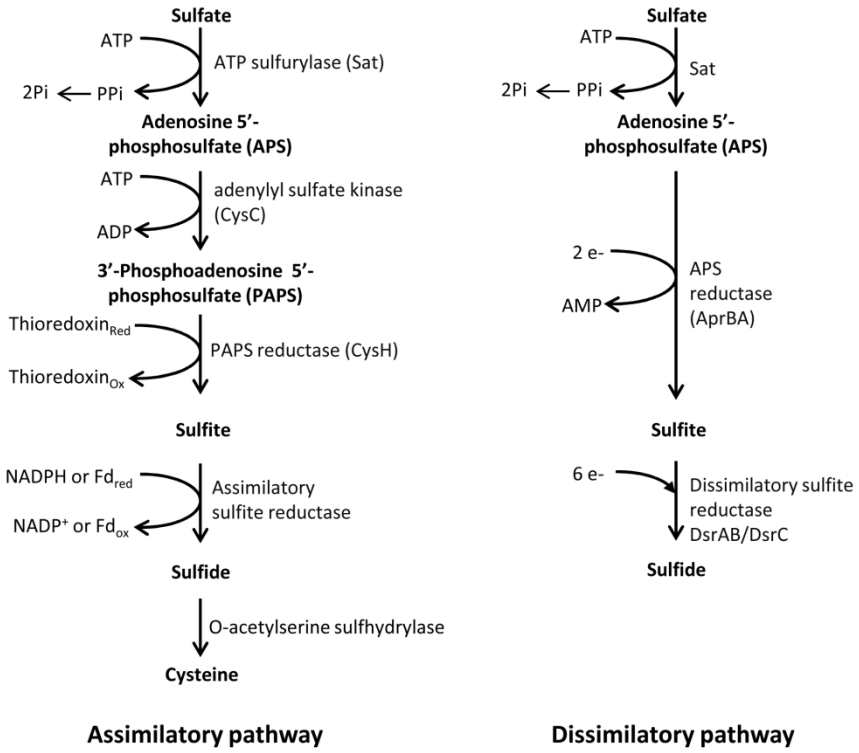

Figure 2.5 - Schematic representation of the prokaryotic assimilatory and dissimilatory pathways of sulfate reduction.

The APS reductase is an αβ heterodimer containing an FAD group in the AprA subunit and two $[4Fe\text{-}4S]^{2+/1+}$ clusters in the AprB subunit. AprBA is an example of a modular redox protein, as the AprA subunit shows strong structural similarity (although low sequence identity) to the module/family of flavoproteins containing fumarate reductase and aspartate oxidase, and AprB includes a domain similar to the bacterial ferredoxin module (Fritz *et al.* 2002b). The *aprA* and *aprB* genes share a similar evolutionary profile resulting from vertical inheritance and concurrent LGT. Several *aprBA* genes of SRO were acquired by LGT,

namely among members of the *Syntrophobacterales, Thermodesulfobacterium*, *Thermodesulfovibrio*, *Archaeoglobus* and some deltaproteobacterial lineages (Friedrich 2002; Meyer and Kuever 2007b). The *aprBA* of SOB diverge into two phylogenetic lineages in which one, represented by AprBA from *Allochromatium vinosum*, is the authentic SOB group (lineage I, congruent with the monophyletic DsrAB phylogeny), and the other, represented by AprBA from *Chlorobium tepidum* (lineage II, discordant with DsrAB phylogeny) was acquired by LGT from SRO (Meyer and Kuever 2007a; Meyer and Kuever 2007b). These two lineages correlate with different gene organizations (Figure 2.6) and different physiological partners of AprBA, which are the integral membrane protein AprM in the case of SOB lineage I, and the QmoABC membrane complex (Pires *et al.* 2003) in the case of SRO and SOB lineage II (Meyer and Kuever 2007a; Meyer and Kuever 2007b). This is further supported by homology modeling of AprBA from the two groups, which suggests different interacting partners for AprB (Meyer and Kuever 2008).

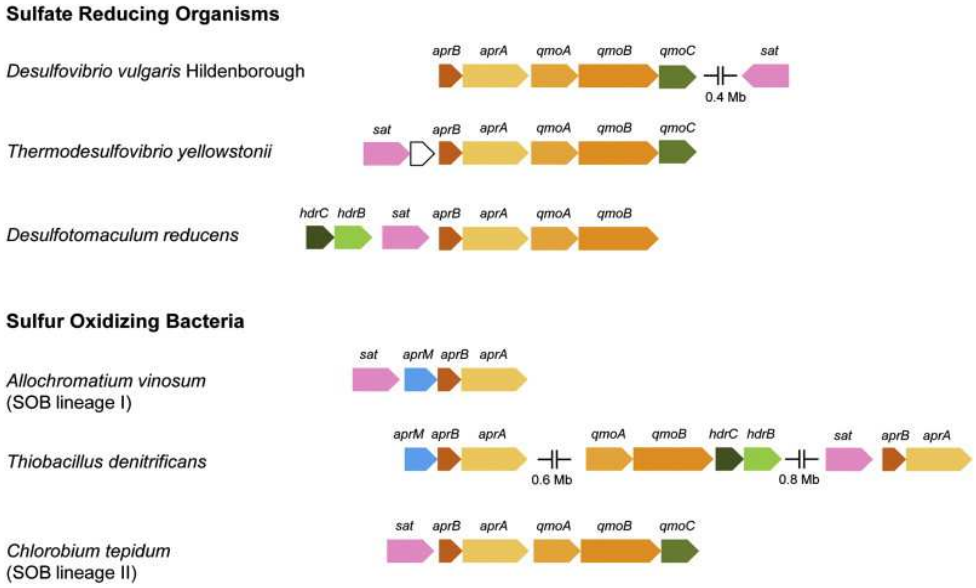

Figure 2.6 - Genomic organization of the *sat*, *apr* and *qmo* genes in selected SRO and SOB. *sat*, ATP sulfurylase; *aprBA*, APS reductase; *aprM*, transmembrane protein; *qmoABC*, subunits of the Qmo complex; *hdrBC*, subunits of heterodisulfide reductase. Adapted from (Meyer and Kuever 2007a; Meyer and Kuever 2007b).

2.2.4 - MODULARITY OF SIMPLE RESPIRATORY MEMBRANE COMPLEXES

The modular nature of redox proteins is particularly evident in membrane-associated respiratory complexes. The simplest family of such complexes is the Complex Iron-Sulfur Molybdoenzyme family that operates on a variety of reducing or oxidizing substrates, including formate, nitrate and several sulfur compounds (thiosulfate, DMSO, polysulfide and tetrathionate) (Rothery *et al.* 2008; Magalon *et al.* 2011). This family is widespread in bacteria and greatly contributes to the flexibility of their respiratory chains (Richardson 2000; Rothery *et al.* 2008). Phylogenetic analysis indicates that most members of this family are very ancient and were likely present in LUCA (Schoepp-Cothenet *et*

al. 2012). CISM proteins include three subunits, or redox modules: i) a catalytic subunit that binds a pterin-guanine dinucleotide cofactor (that includes either Mo or W), and an [Fe-S] cluster; ii) a four-cluster subunit that binds four $[4Fe\text{-}4S]^{2+/1+}$ centers and is responsible for electron transfer between the membrane and catalytic subunits; and iii) a membrane subunit that has a quinone-binding site and is responsible for anchoring the other subunits to the membrane and for quinol oxidation/quinone reduction (Rothery *et al.* 2008) (Figure 2.4B). The quinone-interacting membrane subunit is the one showing more variation, and it can be broadly divided in two families: the first one comprises smaller proteins with 4 or 5 transmembrane helices (TMH), as in the case of FdnI of formate dehydrogenase and NarI of nitrate reductase, respectively. This family, which is usually referred to as the NarI-family, binds two hemes *b* on opposite sides of the membrane (Berks *et al.* 1995; Jormakka *et al.* 2002; Bertero *et al.* 2003). The hemes are coordinated by histidines present in two TMH in the case of NarI and three in the case of FdnI. The second family, which is usually referred to as the NrfD/PsrC family, includes between 8 and 10 TMH (Jormakka *et al.* 2008; Simon *et al.* 2008). Sequence alignments indicate no conserved histidines to serve as heme ligands, and the structurally characterized member of this family (PsrC) does not contain hemes (Jormakka *et al.* 2008). However, heterologous production of another protein from this family (DsrP from *A. vinosum*) unexpectedly resulted in a heme *b*-containing protein (Grein *et al.* 2010). Therefore, it cannot be excluded that some members of this family may bind hemes.

The archetypal trimeric organization including a membrane anchor protein, an electron transfer subunit and a catalytic subunit is also found in a variety of other respiratory enzymes such as membrane-bound uptake hydrogenases, succinate dehydrogenases/fumarate reductases and others (Cecchini *et al.* 2002; Baymann *et al.* 2003; Vignais and Billoud 2007). In many cases these membrane complexes are involved in energy conservation through charge separation and redox loops (Jormakka *et al.* 2003; Simon *et al.* 2008). In SRO several respiratory membrane complexes are variations of this archetypal organization and have a specific role in dissimilatory sulfur metabolism. Two examples are the QmoABC and DsrMKJOP (previously described in Section 2.1), which are strictly conserved in SRO and are physiological partners of the two terminal reductases AprBA and DsrAB. These complexes are also present in other organisms that dissimilate sulfur compounds such as SOB and, in the case of the Dsr complex, in sulfite/thiosulfate/organosulfonate reducers, so they seem to have a dedicated role in sulfur metabolism. An interesting feature of QmoABC and DsrMKJOP is that they both contain subunits that are closely related to subunits of heterodisulfide reductases (Hdr), as already discussed. The second group of SRO membrane complexes, which includes Qrc and the Hmc/Tmc/Nhc family, are specific for those SRO that are rich in multiheme cytochromes *c* (mainly of the *Deltaproteobacteria* class).

2.2.5 - CYTOCHROME *C*-ASSOCIATED MEMBRANE COMPLEXES OF DELTAPROTEOBACTERIAL SRO

The presence of TpIc_3 in SRO correlates also with the presence of several membrane redox complexes having a periplasmic cytochrome *c* subunit. These complexes have also a highly modular character, as discussed above, and they are either involved in quinone reduction (Qrc and Nhc) or transmembrane electron transfer (Tmc and Hmc). They accept electrons from the TpIc_3 and/or seem to be involved in syntrophic metabolism. Here, only the QrcABCD complex will be described next.

2.2.5.1- THE QRCABCD COMPLEX

The membrane-associated Quinone Reductase Complex (Qrc) was first described as a molybdopterin oxidoreductase involved in H_2 oxidation, by screening a library of *Desulfovibrio alaskensis* G20 transposon mutants for strains deficient in syntrophic growth with a methanogen (Li *et al.* 2009). Three mutants were identified with mutations in the *cycA* gene (TpIc_3), *hydB* ([FeFe] hydrogenase) and *mopB* (coding for a putative molybdopterin oxidoreductase). The *cycA* and *mopB* mutants were also impaired in their ability to grow with H_2 or formate (but not lactate) as electron donors for sulfate reduction, pointing to their involvement in the electron transfer chain from H_2 or formate to sulfate (Li *et al.* 2009). The Qrc complex was isolated from *D. vulgaris* Hildenborough, where it was shown to act as a TpIc_3:menaquinone oxidoreductase, but not to be a molybdopterin oxidoreductase, as it lacks a molybdenum or tungsten cofactor (Venceslau *et al.* 2010).

The Qrc complex is composed of four subunits, three periplasmic (QrcABC) and one integral membrane subunit (QrcD) (Figures 2.4). QrcA is a membrane-anchored hexa- or pentaheme cytochrome *c*, QrcB is a membrane-anchored protein of the molybdopterin oxidoreductase family, but which does not contain a molybdopterin cofactor. QrcC is a four cluster protein and QrcD is an integral membrane protein of the NrfD/PsrC family. The three QrcBCD subunits are analogous to the three subunits of CISM complexes discussed above. Thus, Qrc is an interesting variation of the CISM family that includes additionally a cytochrome *c* subunit (Venceslau *et al.* 2010). In addition, its subunits are also closely related to some subunits of the Alternative Complex III (Act), which performs the reverse reaction of oxidizing the quinone pool and reducing a periplasmic redox partner (Yanyushin *et al.* 2005; Pereira *et al.* 2007b; Gao *et al.* 2009). Like Qrc, the Act has a subunit related to molybdopterin oxidoreductases, which lacks a molybdopterin cofactor, as also observed for the Nqo3/NuoG subunit of Complex I (Sazanov and Hinchliffe 2006). The function of this protein in Qrc is presently unknown, as it is also the case for its homologues in Act and Nuo complexes, and it may have only a structural role. The *D. vulgaris* QrcABCD complex contains six hemes *c*, one $[3Fe\text{-}4S]^{1+/0}$ cluster and three $[4Fe\text{-}4S]^{2+/1+}$ (Venceslau *et al.* 2010), whose redox potentials were determined by EPR (Venceslau *et al.* 2011). The Qrc complex is efficiently reduced by periplasmic hydrogenases and formate dehydrogenases only in the presence of TpIc_3, and can reduce menaquinone analogues, having activity as TpIc_3:menaquinone oxidoreductase (Venceslau *et al.* 2010). Thus, Qrc constitutes the missing link between TpIc_3 and the quinone pool. The *qrcABCD* genes are present

in *Deltaproteobacteria* SRO that have TpIc_3 and hydrogenases or formate dehydrogenases lacking a membrane subunit for direct quinone reduction (Venceslau *et al.* 2010; Pereira *et al.* 2011). The fact that it is essential for growth of *D. alaskensis* G20 in H_2 or formate and sulfate (Li *et al.* 2009), indicates that Qrc is the physiological electron acceptor of the TpIc_3 in this organism, and cannot be replaced by other complexes such as Tmc and Hmc, which are also present in this organism. Furthermore, Qrc also seems to be implicated in syntrophic growth of this organism (Li *et al.* 2009; Li *et al.* 2011) and also *D. vulgaris* (Walker *et al.* 2009). In *D. vulgaris*, Qrc forms a supramolecular complex with the TpIc_3 and a periplasmic hydrogenase (Venceslau *et al.* 2011). The quinone binding site in QrcD is located close to the [3Fe-4S]$^{1+/0}$ cluster of QrcC (Venceslau *et al.* 2010). Energy conservation by QrcABCD will depend on whether proton uptake for quinone reduction occurs on the periplasmic side of the membrane (electroneutral process), or from the cytoplasm (electrogenic process) as it has been proposed for PsrC (Jormakka *et al.* 2008). We have suggested that the Qrc and Qmo complexes may be involved in a redox loop mechanism that sustains electron transport across the membrane to the cytoplasmic reduction of sulfate, coupled to proton motive force generation during sulfidogenic growth on H_2 or formate (Venceslau *et al.* 2010).

The evolutionary relationship between Qrc, CISM complexes and Act is an interesting issue that deserves further study. Qrc may have evolved from a CISM complex by association of a cytochrome *c* and loss of the molybdopterin cofactor. Yanyushin *et al.* have also proposed that the Act complex arose from Qrc by acquisition of additional subunits (Yanyushin

et al. 2005), which would place Qrc as a stepping stone in evolution of bacterial complexes. Whatever the case, Qrc is an excellent example of how a different function can be achieved with a minimal modification of subunits, a strategy that forms the basis for the diversity and flexibility of bacterial energy metabolism.

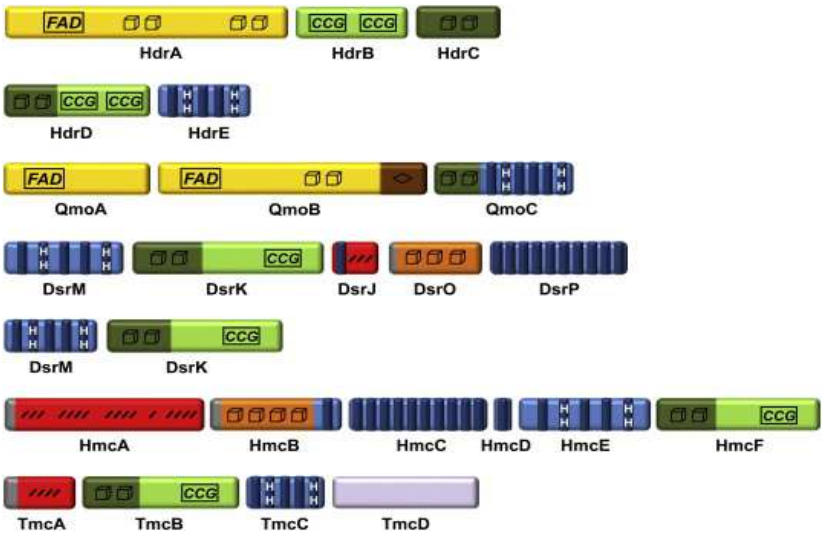

Figure 2. 7 - Schematic representation of Hdr proteins and related complexes from SRO. Similar colors denote sequence identity. Cubes – [4Fe-4S] clusters; CCG – $CX_nCCGX_mCX_2C$ sequence motif; transmembrane heleces are in dark blue; signal peptide in grey, H – conserved histidines; and / - hemes *c*.

2.2.6 - HDR-RELATED PROTEINS AS WIDESPREAD REDOX MODULES IN ANAEROBIC RESPIRATION

In the previous section the genomic analysis of energy metabolism genes in SRO described a very high number of genes related to heterodisulfide reductases, which has also been pointed out by other authors in the

context of individual genomes (Klenk *et al.* 1997; Spring *et al.* 2009; Strittmatter *et al.* 2009; Junier *et al.* 2010; Callaghan *et al.* 2012), or in other classes of organisms such as the acetogenic *Moorella thermoaceticum* (Pierce *et al.* 2008). The abundance of Hdr-like proteins in SRO (Mander *et al.* 2002; Pires *et al.* 2003; Mander *et al.* 2004; Pires *et al.* 2006; Pereira *et al.* 2007a; Strittmatter *et al.* 2009; Junier *et al.* 2010; Pereira *et al.* 2011) may indicate they were present in ancestral organisms, and/or that there was substantial exchange of genetic material between methanogens and SRO (and other classes of organisms), which could be due to their sharing common habitats. Hdrs are representative enzymes of a group of quite widespread proteins responsible for reduction of disulfides or oxidation of thiols (Hedderich *et al.* 1999), but they belong to a larger family that includes proteins that seem to have other functions (see below). In methanogenic archaea, the heterodisulfide is not an external substrate, but is produced in the final step of methanogenesis. By analogy, it is possible that thiol/disulfides may be generated in other anaerobes and be involved in the respiratory chain, which would suggest that a sulfur-based energy metabolism, of obvious ancient origin, could be more widespread than presently considered (Hedderich *et al.* 1999; Martin 2011).

There are two types of Hdr enzymes (Thauer *et al.* 2008): in methanogens without cytochromes a soluble HdrABC is present (Hedderich *et al.* 2005), which forms a complex with the MvhADG hydrogenase. This complex couples the endergonic reduction of ferredoxin by H_2 with the exergonic reduction of the heterodisulfide by H_2, through an electron bifurcation process (Kaster *et al.* 2011). In

methanogens with cytochromes, a membrane-bound enzyme is present, HdrDE, which uses the quinone-like cofactor methanophenazine as electron donor in a process coupled to energy conservation (Kunkel *et al.* 1997; Ide *et al.* 1999; Deppenmeier 2004). The key subunits in Hdrs are the catalytic subunits (HdrB in the soluble enzyme and HdrD in the membrane-bound enzyme; actually HdrD resembles a fusion of the HdrBC proteins), and the HdrA subunit that contains an FAD group presumed to be responsible for bifurcation of electrons coming from the Mvh hydrogenase. There are several proteins related to both HdrA and HdrD in the genomes of SRO (Pereira *et al.* 2011). Overall, both HdrA and HdrD (or more precisely the CCG domain) can be considered as additional modules of the "redox construction kit". Here, only the HdrA-related proteins will be described next.

2.2.6.1- HDRA-RELATED PROTEINS

A complete set of *hdrABC* genes is found in many SRO, either next to a set of *mvhDGA* genes for an Mvh [NiFe] Hase, or next to a set of *floxABCD* genes (for *fl*avin *ox*idoreductase) as described in the previous section (see Table 2.3). In many cases the *hdrBC* genes are absent (*hdrA-mvhDGA* or *hdrA-floxABCD* sets). We proposed that these proteins are part of electron-transfer pathways from oxidation of H_2 or ethanol involving energy coupling through electron bifurcation. A group of multidomain HdrA-like proteins was defined by Strittmater *et al.* as HdrL. These are large proteins containing an NADH binding site and, in some cases, a fumarate reductase domain (Strittmatter *et al.* 2009). With a few exceptions, they are restricted to the sulfate/sulfite reducing

Deltaproteobacteria and *Firmicutes*. It is noteworthy that some of the HdrL (and HdrA) proteins contain selenocysteine, and that there is a conserved CxxCxxCxxCxxCxxxC motif of unknown function present in all available HdrL sequences. The *hdrL* genes are usually found in loci together with *hdrA* and genes coding for a formate dehydrogenase or a pyruvate:ferredoxin oxidoreductase (Pereira *et al.* 2011), indicating that pyruvate and formate may serve as electron donors for reduction of HdrA/L. Often an *mvhD* gene is found next to *hdrA* or fused to it (as also observed in QmoB).

Hdr proteins are also noteworthy in the genome of the acetogenic bacterium *M. thermoacetica* (Pierce *et al.* 2008) where they are present in four gene loci including three HdrL proteins. One is an *hdrABC* locus, the other includes the *hdrLBC* genes next to the acetyl-CoA synthase (*acs*) genes, the third is an *hdrLBC-floxABCD* cluster and finally there is an *hdrDL* locus, where *hdrD* shows high similarity to *hmcF*.

HdrA and HdrD modules are also present in the Benzoyl-coenzyme A reductase complex BamBCDEFGHI, present in several anaerobes capable of degrading aromatic compounds (Wischgoll *et al.* 2005; Kung *et al.* 2009; Loffler *et al.* 2011). This large complex includes the active site subunit BamB, which contains a tungstopterin cofactor, and the iron-sulfur protein BamC that shows similarity to the electron transfer subunit of hydrogenases. BamD and BamE are HdrD- and HdrA-like proteins, while BamF shows similarity to MvhD and contains selenocysteine. BamGHI are similar to the soluble components of Complex I (Wischgoll *et al.* 2005). The BamBCDEFGHI complex is another striking example of the highly modular character of redox proteins, in

this case with a quite intricate arrangement that suggests a complex mechanism.

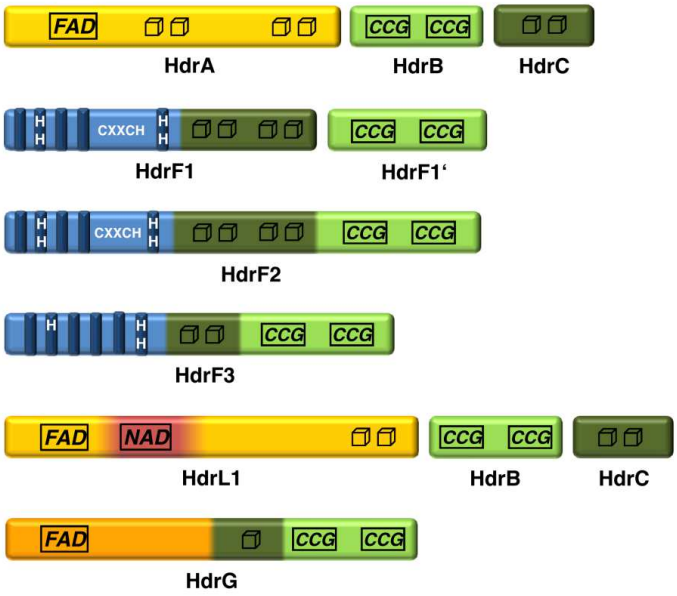

Figure 2. 8 - Schematic representation of Hdr-related proteins. Similar colors denote sequence identity. Cubes—[4Fe-4S] clusters, CCG—CXnCCGXmCX2C sequence motif, transmembrane helices are in dark blue, H—conserved histidines, and CXXCH—possible heme c binding sequence.

2.2.7 - Conclusions

The modular nature of respiratory proteins is well apparent in proteins from SRO. In particular, several membrane-associated redox complexes from these organisms present new and interesting variations of the typical trimeric arrangement of simple respiratory proteins (catalytic, electron transfer and membrane anchor/quinone binding subunits). These variations are also reflected in the fact that the SRO complexes do not act directly on organic/inorganic substrates, as observed in the CISM family, but rather interact with other redox proteins, which considerably

complicates *in vitro* studies and elucidation of their bioenergetic mechanisms. The SRO membrane complexes have a dedicated role in sulfur metabolism as they are also found in many SOB and organisms dissimilating other sulfur compounds, such as sulfite, thiosulfate and organosulfonates. Several of the proteins involved are related to subunits of heterodisulfide reductases of methanogenic archaea, which probably reflects a common ancient origin of sulfur-metabolizing organisms and methanogens and their close environmental association.

A subset of SRO, mainly of the *Deltaproteobacteria*, relies on multiheme cytochromes *c* as additional redox modules to diversify their respiratory metabolism. The prototype protein is the TpIc_3 that functions as a hub in periplasmic electron transfer pathways, with links to several membrane complexes having also a cytochrome *c* subunit. One of these complexes, QrcABCD, is closely related to the CISM family and seems to be a cross-point in the evolution of bacterial complexes. It is an excellent example of how a different function can be achieved with a minimal modification of subunits.

Finally, the Hdr proteins, namely HdrA, seem to be a model protein for a larger family with a wide distribution. The function of many of these proteins is still unknown, but their similarity to Hdrs may suggest that sulfur-based metabolic pathways may be more widespread than presently considered.

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CHAPTER 3

STUDIES OF THE PHYSIOLOGICAL ROLE OF A CONSERVED MEMBRANE-BOUND COMPLEX IN SRP: THE QMOABC COMPLEX FROM *DESULFOVIBRIO* SP.

CHAPTER 3

SECTION 3.1

THE MEMBRANE QMOABC COMPLEX INTERACTS DIRECTLY WITH THE DISSIMILATORY ADENOSINE 5´-PHOSPHOSULFATE REDUCTASE IN SULFATE REDUCING BACTERIA

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3.1.1 - SUMMARY

The adenosine 5'-phosphosulfate reductase (AprBA) is the enzyme responsible for the reduction of adenosine 5'-phosphosulfate (APS) to sulfite in the biological process of dissimilatory sulfate reduction, which is carried out by a ubiquitous group of sulfate reducing prokaryotes. The electron donor for AprBA has not been clearly identified, but was proposed to be the QmoABC membrane complex, since an *aprBA-qmoABC* gene cluster is found in many sulfate-reducing and sulfur-oxidising bacteria. The QmoABC complex is essential for sulfate reduction, but electron transfer between QmoABC and AprBA has not been reported. In this work we provide the first direct evidence that QmoABC and AprBA interact in *Desulfovibrio* spp., using co-immunoprecipitation, cross-linking Far-Western blot, tag-affinity purification and surface plasmon resonance studies. This showed that the QmoABC-AprBA complex has a strong steady-state affinity (K_D = 90 ± 3 nM), but has a transient character due to a fast dissociation rate. Far-Western blot identified QmoA as the Qmo subunit most involved in the interaction. Nevertheless, electron transfer from menaquinol analogues to APS through anaerobically purified QmoABC and AprBA could not be detected. We propose that this reaction requires the involvement of a third partner to allow electron flow driven by a reverse electron bifurcation process i.e. electron confurcation. This process is deemed essential to allow coupling of APS reduction to chemiosmotic energy conservation.

3.1.2 - INTRODUCTION

Sulfate respiration is an anaerobic process carried out by a phylogenetically diverse group of organisms including both Bacteria and Archaea. This process is a major contributor to the global cycling of sulfur and carbon in anaerobic habitats, and has very important environmental and economical impacts (Muyzer and Stams 2008; Barton and Fauque 2009). Sulfate-reducing prokaryotes (SRP) are found ubiquitously in anaerobic environments, and are particularly abundant in marine habitats due to the high concentration of sulfate in sea water. As a group SRP are physiologically versatile and capable of metabolizing a wide variety of substrates, and they can also grow syntrophically with other organisms in the absence of sulfate (Stams and Plugge 2009; Plugge *et al.* 2011). Despite its fundamental importance, the mechanism of energy conservation in sulfate respiration remains to be fully elucidated. For many years it was thought that quinones did not play a role in the process, despite their known presence in SRP, and intracellular hydrogen cycling was proposed to account for proton motive force generation. Nowadays, hydrogen cycling is considered as only one of the possible pathways for energy conservation, operating in some, but not all SRP (Keller and Wall 2011; Pereira *et al.* 2011). Sulfate reduction is an intracellular process requiring active transport of sulfate, and its activation by reaction with ATP to form adenosine 5´-phosphosulfate (APS). The two terminal reductases, APS reductase (AprBA) and dissimilatory sulfite reductase (DsrAB), are soluble and thus not directly involved in membrane-linked electron transport. One of the key questions remaining about sulfate reduction is the identification of

the electron donors to AprBA and DsrAB. The involvement of membrane proteins in the process was first described by Mander *et al.* and Pires *et al.* through the identification of the DsrMKJOP (initially named Hme) and QmoABC complexes (Mander *et al.* 2002; Pires *et al.* 2003). These two complexes are found both in SRP (Pereira 2008) and in many anoxygenic phototrophic and chemotrophic sulfur-oxidizing bacteria (SOB) (Frigaard and Dahl 2009), indicating a dedicated role in sulfur metabolism. Furthermore, the two complexes are conserved in the genomes of SRP described to date, with very few exceptions: the archaeon *Caldivirga maquilingensis* lacks the *qmoABC* genes and in some Gram-positive bacteria the *qmoC* gene is absent; in both these organisms a simpler version of the DsrMKJOP complex occurs since only the *dsrMK* genes are present (Junier *et al.* 2010; Pereira *et al.* 2011). The QmoABC and DsrMKJOP complexes share an interesting characteristic in that they both contain subunits that are related to heterodisulfide reductases (Hdr) of methanogens (Thauer *et al.* 2008), and subunits known to interact with quinones. In several organisms, the *qmoABC* genes cluster with the *aprBA* genes, and the *dsrMKJOP* genes cluster with *dsrAB*, strongly suggesting an involvement of QmoABC in the electron transfer pathway to AprBA and DsrMKJOP in the electron transfer pathway to DsrAB.

The QmoABC complex has one membrane (QmoC) and two cytoplasmic subunits (QmoAB), and the two QmoC hemes *b* are reduced by quinols, indicating that the Qmo complex participates in electron flow between the quinone pool and the cytoplasm, in a process that may result in energy conservation (Pires *et al.* 2003). In *Desulfovibrio vulgaris*

Hildenborough a deletion mutant of the *qmoABC* genes could not grow with sulfate as electron donor, but grew normally with sulfite or thiosulfate, providing conclusive evidence that QmoABC is required for reduction of sulfate (Zane *et al.* 2010). Also, in the green sulfur-oxidizer *Chlorobium tepidum* the Qmo complex was shown to be involved in oxidation of sulfite as an intermediary in the sulfur oxidation pathway (Chan *et al.* 2008; Rodriguez *et al.* 2011). These results show that the Qmo complex is involved in electron flow between the menaquinone pool and APS reduction or oxidation by AprBA. However, direct electron transfer between the isolated *Desulfovibrio desulfuricans* ATCC 27774 Qmo complex and AprBA could not be detected, which could indicate that additional proteins are involved in the pathway (Pires *et al.* 2003). In this work we report protein-protein interaction studies that show that there is a direct interaction between QmoABC and AprBA, and that the interaction involves the QmoA subunit. The mechanism of AprBA reduction is further discussed.

3.1.3 - Materials and Methods

3.1.3.1 - Protein purification

Cells of *D. desulfuricans* ATCC 27774 were grown according to (Liu and Peck Jr. 1981). The cells were broken and centrifuged and the membrane fraction was used to purify the QmoABC complex in n-Dodecyl-β-D-maltoside (DDM), as previously described by (Pires *et al.* 2003), following its characteristic UV-Visible absorption spectrum (Pires *et al.* 2003). The purification of Qmo was carried out both in aerobic and

anaerobic conditions. AprBA was purified from the soluble fraction in anaerobic conditions following the catalytic activity of sulfite oxidation (Fritz *et al.* 2002a; Fritz *et al.* 2002b). Anaerobic purifications were carried out inside a Coy anaerobic chamber (95% N_2, 5% H_2) using an AKTA^{TM} Prime plus^{TM} system. The soluble fraction from *D. desulfuricans* was ultracentrifuged at 140,000 x *g* for 2 h, and then applied to a Q-Sepharose FF column equilibrated with 50 mM Tris-HCl (pH 7.6) buffer with 10% glycerol (v/v) (buffer A). A stepwise gradient of increasing NaCl concentration was performed and fractions were separated according to UV-Visible spectra. The fractions containing highest AprBA activity, which eluted between 180-200 mM NaCl, were pooled. After concentration and lowering of ionic strength, this sample was loaded on a Q-Sepharose HP column equilibrated with buffer A. Again, a stepwise gradient of increasing NaCl concentration was performed. The fractions were separated according to the UV-Visible spectra and activity. The pool of fractions with higher activity was diluted in buffer A and applied in a second Q-Sepharose HP column equilibrated with 10 mM potassium phosphate buffer (pH 7) containing 10% glycerol (v/v) (Buffer B). A stepwise gradient of increasing NaCl concentration was performed, and fractions containing purified AprBA eluted at 150 mM NaCl. The purified enzyme had a sulfite oxidation activity of 3.3 µmol min^{-1} $mg^{-1}$and displayed the characteristic two subunits on an SDS-PAGE gel.

3.1.3.2 - APS REDUCTASE ACTIVITY

The AprBA activity was determined as formation of APS in 50 mM Tris-HCl (pH 7.6), 2 mM Na_2SO_3, 2 mM AMP, 1 mM $K_3Fe(CN)_6$, at room

temperature (Fritz *et al.* 2000; Fritz *et al.* 2002a), or by APS reduction in 80 mM potassium phosphate (pH 7), 30 µM APS and 0.75 mM methyl viologen as reductant (Fritz *et al.* 2002a). Methyl viologen was reduced with 0,2 g of metallic zinc granules in the same buffer.

3.1.3.3 - CO-IMMUNOPRECIPITATION

Antibodies for QmoABC complex and AprBA from *D. desulfuricans* were produced from the purified proteins by Davids Biotechnology (Regensburg, Germany) and used for Co-immunoprecipitation (Co-IP) experiments with the Thermo Scientific Pierce® Co-IP kit, following the kit instructions. The anti-Qmo antibody did not cross-react with AprBA, and the anti-Apr antibody did not cross-react with QmoABC. Two approaches were used to investigate protein-protein interaction, one based on Anti-QmoABC antibodies and the other based on Anti-AprBA antibodies. In the first case, 500 µg of Anti-QmoABC antibody was immobilized in the AminoLink® Plus Coupling Resin in a small column, and 1 µM of Qmo in the kit Lysis/Wash buffer was added and incubated for 1h at 4 ^{o}C. After one washing step with Lysis/Wash buffer 1 µM of AprBA in the same buffer was loaded in the column and incubated for 2h at 4 ^{o}C. After five washing steps, the co-IP products were eluted with the kit Elution buffer. The protocol was repeated with 500 µg of Anti-AprBA antibody, 1 µM of AprBA and 1 µM of QmoABC in the same buffer. Control experiments were run in parallel with no antibody bound to the control resin. The eluates (~100 µg) were separated in SDS-PAGE gels (12% acrylamide, (v/v)), and transferred to polyvinylidene difluoride (PVDF) membranes (Transfer buffer: 48 mM Tris-HCl pH 9.2 and 39 mM

Glycine) using a Mini Trans-Blot® SD semi-dry electrophoretic transfer cell (Bio-Rad) during 40 minutes at 4 ^{o}C, 100 V and 350 mA. The membranes were blocked with blocking buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20 (v/v) and 5% non-fat milk (w/v)), overnight at room temperature. After two washing steps with TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20 (v/v)) anti-QmoABC antibody at 1:200 dilution in TBST or anti-AprBA antibody at 1:1 000 dilution in TBST were incubated with the membranes for 1 h, followed by two washing steps with TBST, and incubation with anti-rabbit IgG antibody (Sigma-Aldrich®) at 1:15 000 dilution in TBST for 45 minutes. After three washing steps with TBS, protein detection was performed with Alkaline Phosphatase Buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) and NBT (nitro-blue tetrazolium chloride) /BCIP (5-bromo-4-chloro-3-indolyl phosphate).

3.1.3.4 - SURFACE PLASMON RESONANCE

The surface plasmon resonance (SPR) experiments were performed at 25 ^{o}C on a BIAcore 2000 instrument (Biacore Inc., GE HealthCare). The proteins samples were exchanged to the buffer used as running buffer for the SPR experiments (10 mM HEPES pH 7.4 + 150 mM NaCl + 3 mM EDTA + 0.01% DDM (w/v)), using a HiTrap™ Desalting column (Amersham Biociences). AprBA was immobilized in a CM5 sensor chip (GE® Healthcare) by standard NHS/EDC amine coupling resulting in an immobilization level of 1000 RU. Flow cell 1 was similarly treated with buffer in the absence of AprBA (control cell). Interaction experiments with QmoABC were performed with duplicate injections of 3.9, 7.8, 15.6,

31.25, 62.5 nM of QmoABC at a flow rate of 15 µl/min. After the end of each injection dissociation was performed with running buffer for 10 min, after which all of the protein completely dissociated from the surface (as indicated by a return to baseline level of the sensorgram) and thus no further regeneration was required. The sensorgrams were processed using the double referencing method to eliminate the nonspecific binding from background contribution and the buffer artifacts were removed by subtracting signals from the reference flow cell and from buffer blank injections. The BIAevaluation 3.2 RC1 analysis software was used to determine k_a and k_d from the processed data sets by globally fitting to a 1:1 biomolecular binding model with drifting baseline. The K_D was calculated from the quotient k_d/k_a.

For the competition experiments, 62.5 nM of QmoABC was incubated with 62.5 nM or 125 nM AprBA and injected in the chip surface at the same flow rate as before.

3.1.3.5 - CROSS-LINKING FAR-WESTERN BLOT

10 µg of pure QmoABC was separated in a 12% SDS-PAGE gel and blotted to a PVDF membrane. After overnight blocking, the membrane was incubated with AprBA (1µM) for 1 h in 20 mM Tris-HCl pH 7.6, 10% Glycerol (v/v), at room temperature. The membrane was washed once with bidistilled water (bDW) and incubated with 32 mM N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Sigma-Aldrich®) in bDW for 1 h at room temperature (Sato *et al.* 2011). After three washing steps with bDW, Western Blot against Anti-AprBA was

performed. As positive control we used AprBA and as negative control QmoABC that was not incubated with AprBA.

3.1.3.6 - *D. VULGARIS* HILDENBOROUGH STRAINS AND GROWTH CONDITIONS

The strains used in this part of the work are listed in Table 3.1 and the primers used for the plasmid construction are listed in Table 3.2. A mutant strain lacking the *qmoA* gene was produced by double homologous recombination in *D. vulgaris* Hildenborough - IPAR02 - according to (Keller *et al.* 2011), with the exception that following electroporation the cells were recovered and plated in MOYLS3 (lactate 30 mM/sulfite 15 mM) and the electroporation parameters were 1500V, 250 Ω, and 25 µF. The pMOIP02 plasmid for the *qmoA* deletion was obtained by sequence ligation independent cloning (SLIC) (Li and Elledge 2007). Three segments were amplified by PCR with Herculase polymerase II (Stratagene®): 942 bp upstream of *qmoA* (primers #1 and #2), 932 bp downstream of *qmoA* (primers #3 and #4) and the kanamycin resistance gene from pSC27 (Keller *et al.* 2011) (primers #5 and #6); and then added into pMO719 background via SLIC. Products from the amplifications were transformed into *E. coli* α-select Silver Efficiency (Bioline®) and successful transformants were isolated on LC medium (Zane *et al.* 2010) containing 100 µg/ml spectimonycin and/or 50 µg/ml kanamycin.

Table 3.1 - List of bacteria strains and plasmids used in this work.

Strain or plasmid	Genotype or relevant characteristics	Source and/ or reference
E. soli strains		
α-Select (Silver efficiency)	*F⁻ deoR endA1 relA1 gyrA96 hsdR*17($r_k^- m_k^+$) *supE*44 *thi*-1 Δ(*lacZYA-argFV*169) Φ*80δlacZ*ΔM15 *λ*⁻	Bioline
***D. vulgaris* strains**		
ATCC 29579	WT *D. vulgaris* Hildenborough	ATCC
IPAR02	WT Δ*qmoA*::Km^R	This work
IPAR03	WT Δ*qmoA*::Km^R + pMOIP05	This work
Plasmids		
pMO719	pCR8/GW/TOPO containing SRB replicon (pBG1); $Spec^R$	Keller et al 2011
pSC27	*Desulfovibrio* shuttle vector; source of *aph(3')-II*; Km^R	Keller et al 2011
pMO9075	pCR8/GW/TOPO containing SRB replicon (pBG1), Km^r gene-*aph(3')-II* promoter, multicloning site; Sp^r	Keller et al 2011
pSLDV0171	pCR8/GW/TOPO with the last 750 bp of DVU0171, Strep-TEV-FLAG-Tag, Km^r gene-aph(3')-II promoter and 750 bp downstream of DVU0171	Chhanbra et al 2011
pMOIP02	pCR8/GW/TOPO with 942 bp upstream and 932 downstream of *aph(3')-II* cassete to delete *qmoA*; Sp^r	This work
pMOIP05	*qmoA* expression vector with STF-Tag, $Spec^R$	This work

Correct isolates were identified by the expected PCR amplicons from the plasmids constructs and also by sequencing performed at the DNA Core Facility at the University of Missouri, USA. The pMOIP02 produced was electroporated into *D. vulgaris* according to (Zane *et al.* 2010; Keller *et al.* 2011), from which strain IPAR02 was obtained, by selecting with

MOYLS3 medium containing 400µg/ml of geneticin. The deletion of *qmoA* was confirmed by Southern blot. The IPAR02 mutant strain grows in lactate/sulfite as described previously for the mutant lacking *qmoABC* (Zane *et al.* 2010) and is kanamycin resistant.

Table 3.2 - List of primers used in this work.

	Primer	Primer sequence (5' → 3')
#1	QmoAUpFwP1	GCC TTT TGC TGG CCT TTT GCT CAC ATA AGA GCG CGG TTC TGA AAT CAT GC
#2	QmoAUpRevP2	CCT GCG TGC AAT CCA TCT TGT TCA ATC ATC CTT GGT ATC CTC CCT ACG TGT
#3	QmoADwnFwP3	CCT TCT ATC GCC TTC TTG ACG AGT TCT TCT AGA CCA TAA TGG CCA GCA GAA TTG G
#4	QmoADwnRevP4	CGA GGC ATT TCT GTC CTG GCT GGA GTG ACG TGT TCA GGA TGA AGG CA
#5	Kan aa2Fw	ATT GAA CAA GAT GGA TTG CAC GCA GG
#6	Kan aa264 Rev	GAA GAA CTC GTC AAG AAG GCG ATA GAA GG
#7	SpecGene-F	CCA GCC AGG ACA GAA ATG CCT CG
#8	pUCoriR	ATG TGA GCA AAA GGC CAG CAA AAG GC
#9	QmoAExpVctrP1Fw	AGG TTG GGA AGC CCT GCA ATG CAG TCC CAG GAG GTA CCA TAT GTC GAA CTC CAT ACT CGT CGT CG
#10	QmoAExpVctrP2Rev	AAT TTT TTC GAA CTG CGG GTG GCT CCA CCT CCC TCT CAC CGT TTG AAT CGC
#11	STF-tag Fw	TGG AGC CAC CCG CAG TTC GAA AAA ATT
#12	STF-tag Rev	GAT CGT GAT CCC CTG CGC CAT CAG ATC CTT GCT ACT TGT CAT CGT CAT CCT TGT AGT CGA TGT CA
#13	SpecRpUC-R	ATG TGA GCA AAA GGC CAG CAA AAG GC
#14	pMO9075-RBS-R	ATG GTA CCT CCT GGG ACT GCA TTG CAG GGC TTC CCA ACC T
#15	pUC ori F	GGC CTT TTG CTG GCC TTT TGC TCA CAT
#16	pMO9075-SLIC-F	CAA GGA TCT GAT GGC GCA GGG

A complementation plasmid pMOIP05 was produced also by SLIC encoding *qmoA* with a Strep-TEV-FLAG (STF) tag. To create this vector

two segments were amplified by PCR: the *qmoA* gene (primers #9 and #10) and the STF-tag gene from pSLIC-DVU0171-STF-Kan-Tag (Chhabra *et al*. 2011a) (primers #11 and #12); and then added into pMO9075 background via SLIC. The amplifications products were transformed into *E. coli* α-select Silver Efficiency (Bioline®), and cells were plated on spectinomycin (100 µg/ml)-containing agar plates. The correct plasmid construct was screened by colony PCR and later confirmed by sequencing at the DNA Core Facility at the University of Missouri, USA.

The pMOIP05 was successfully introduced in IPAR02 by electroporation (Keller *et al.* 2011) selecting with MOYLS3 medium containing 400 µg/ml of geneticin and 100 µg/ml of spectinomycin, to generate the complemented strain IPAR03. The plasmid was confirmed by PCR amplification of the insert and also by sequencing performed in GATC Biotech, Germany. The complemented mutant strain IPAR03 was grown either in MOYLS3 lactate/sulfite medium or MOYLS4 lactate/sulfate medium (Zane *et al.* 2010; Keller *et al.* 2011) with spectinomycin (100 µg/ml).

3.1.3.7 - PULL DOWN ASSAY

For the pull down assay, IPAR03 was grown in 100 ml of MOYLS4 with spectinomycin at 37 ^{o}C for about 24h. Cells were harvested by centrifuging at 2,500 × *g* for 15 minutes at 4 ^{o}C, washed with 20 mM Tris-HCl buffer (pH 7.6) + 10% glycerol (v/v), and again centrifuged as before. Cells were then disrupted using BugBuster® Protein Extraction Reagent (Novagen®) for 20 minutes at room temperature and centrifuged at 16,000 × g for 20 minutes at 4^{o}C. The soluble fraction of IPAR03 was

loaded in micro-columns containing Strep®-Tactin resin (IBA GmbH) equilibrated with 50 mM Tris-HCl pH 7.6, 150 mM NaCl and 10% glycerol (v/v) (Buffer W). After five washing steps with Buffer W, the recombinant protein QmoA was eluted with Buffer W containing 2.5 mM desthiobiotin. The elution product was precipitated in acetone and analyzed by SDS-PAGE and Western Blot with Strep-Tactin horse radish peroxidase (HRP) conjugate. The co-elution of AprBA with QmoA was detected by Western Blot with Anti-AprBA from *D. desulfuricans*. In a control experiment the same conditions were used with wild type cells of *D. vulgaris*.

3.1.3.8 - ELECTRON TRANSFER EXPERIMENTS

The electron transfer between QmoABC and AprBA was tested in spectrophotometer assays inside the anaerobic chamber, using quartz cuvettes equipped with a magnetic stirrer. The first assay was based on AprBA activity, as previously described (Pires *et al.* 2003), following reduction of the menaquinone analogue 2,3-dimethyl-1,4-naphthoquinone (DMN) with sulfite (reverse reaction) at 350 nm (at 270 nm there is interference from AMP). DMN (500 µM) reduction was followed in 50 mM Tris-HCl (pH 7.6) with 0.0125% DDM (w/v), 2 mM Na_2SO_3, 2 mM AMP and 0.5 µM of QmoABC after addition of 1.2 µM AprBA. The second assay was based on oxidation of quinol reduced QmoABC by APS (direct reaction). Qmo (0.3 µM) was reduced with different amounts of menadiol (25, 50, 100, 300, 930 µM) in 10 mM Phosphate buffer (pH 7), 0.0125% DDM (w/v). Qmo heme *b* oxidation

was followed at 424 nm in the presence of different amounts of APS (30, 60 and 120 µM) (Sigma®), after addition of 0.1 µM AprBA.

3.1.4 - RESULTS

A link between the QmoABC complex and APS reductase was first inferred from the co-localisation of their genes in the genomes of several sulfate reducing and sulfur-oxidising bacteria. Subsequent deletion of the *qmo* genes in these organisms proved that the Qmo complex is required for the reduction of sulfate in SRP (Zane *et al.* 2010), and the oxidation of sulfite in green sulfur bacteria (Rodriguez *et al.* 2011). However, the fact that no electron transfer could be observed between the two proteins (Pires *et al.* 2003) raised doubts as to whether there is a direct interaction between them, or if other proteins are involved. Recently, a proteomic study of protein-protein interactions in *D. vulgaris* Hildenborough was reported, in which several key proteins were used as baits for affinity purification followed by mass spectrometry (Chhabra *et al.* 2011b). The bait proteins included *Strep*-tagged AprA and AprB, and again no evidence for a direct interaction with QmoABC proteins was obtained. However, interactions between redox proteins are notably difficult to observe due to their transient nature, which is required for the fast turnover of electron exchange reactions in energy metabolism (Bashir *et al.* 2011; Martinez-Fabregas *et al.* 2011). In addition, the fact that Qmo is a membrane-associated complex is likely to further hinder proteomic-based studies. These kind of high-throughput approaches, although invaluable from the amount of information that can be

obtained, suffer from the use of the same conditions to evaluate many different types of interactions between many different proteins, so a high number of false negative results is likely to occur. In this work we took advantage of the fact that we can purify both QmoABC and AprBA from *D. desulfuricans* ATCC 27774 to perform detailed interaction studies between the two proteins.

3.1.4.1 - CO-IMMUNOPRECIPITATION EXPERIMENTS

The first approach to evaluate a possible interaction between QmoABC and AprBA complexes was to use co-immunoprecipitation (co-IP). For this we used a Thermo Scientific Pierce® Co-IP kit in which the antibodies are covalently coupled to an amine-reactive resin. Anti-QmoABC or Anti-AprBA specific antibodies were generated using the purified proteins, and immobilized in columns containing the coupling resin. The two antibody-loaded resins were then incubated with the corresponding prey protein (QmoABC or AprBA), washed and then incubated with the interacting bait partner (AprBA or QmoABC). After several washing steps the retained proteins were eluted and the Co-IP products were separated by SDS-PAGE and blotted to a PVDF membrane. The membranes were treated by Western blot using the antibodies against the bait protein. Two control experiments were run in parallel, where no antibodies were bound to the resin. The Western blot results (Figure 3.1) show that it was possible to co-immunoprecipitate QmoABC and AprBA, using either of the corresponding antibodies, indicating that there is a direct physical interaction between the two proteins. The control experiments reveal some unspecific retention of both proteins, but the

strong difference between the experiments and the controls are indicative of co-immunoprecipitation.

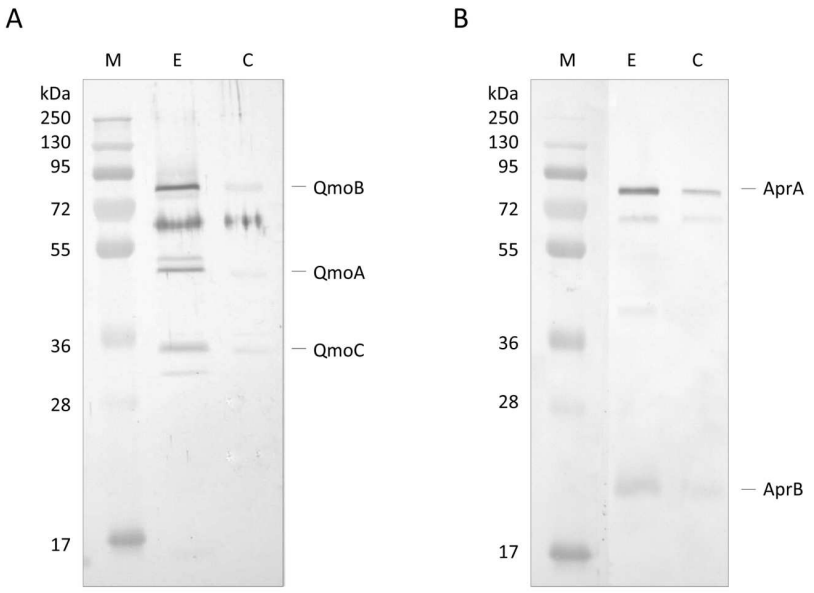


Figure 3.1 - Western Blot analysis of co-immunoprecipitation experiment. (A) Western Blot with Anti-QmoABC of the elution products of: E - the Co-IP using immobilized Anti-AprBA antibody; C - control resin with no antibody. (B) Western Blot with Anti-AprBA of the elution products of: E - the Co-IP using immobilized Anti-QmoABC antibody; C - control resin with no antibody. M – Pre-stained molecular mass markers.

3.1.4.2 - Surface Plasmon Resonance experiments

Since an interaction between QmoABC and AprBA was detected we next sought to quantify the kinetics and affinity parameters of this interaction. For this we used Surface Plasmon Resonance (SPR), which is a gold standard for studying protein-protein interactions, since it can provide direct quantitative measurements of binding kinetics and affinities, without the need for any labeling methods. Using a CM5

sensor chip we tested covalent immobilization of either QmoABC or AprBA. Considerable loss of immobilized material was observed in the case of QmoABC, likely due to gradual dissociation of subunits, whereas this was not observed with immobilized AprBA. Further studies proceeded using AprBA as the ligand and QmoABC as the analyte. An interaction was again observed between the two proteins, which could be detected even at low concentrations of QmoABC. The dissociation of QmoABC was complete after injection stopped, and did not require special regeneration conditions, which confirms the transient nature of the interaction between the two proteins. The sensorgrams obtained (Figure 3.2A) were used to calculate the binding rate constants, by fitting the results to a 1:1 interaction model with drifting baseline, yielding an association rate constant $k_a = (3.0 \pm 0.1) \times 10^5\ M^{-1}s^{-1}$, a dissociation rate constant $k_d = (2.7 \pm 0.4) \times 10^{-2}\ s^{-1}$, and an equilibrium affinity constant $K_D = 90 \pm 3$ nM. These values reveal a high affinity for the AprBA-QmoABC complex in steady-state conditions, and that the complex dissociation is very fast, as it is to be expected for an electron transfer interaction. To further validate these results we carried out a competition assay in which QmoABC was pre-incubated with two different concentrations of free AprBA in solution before the SPR measurement (Figure 3.2B). This experiment confirmed a reduced interaction between QmoABC and the immobilized AprBA protein, due to the competition of AprBA in solution.

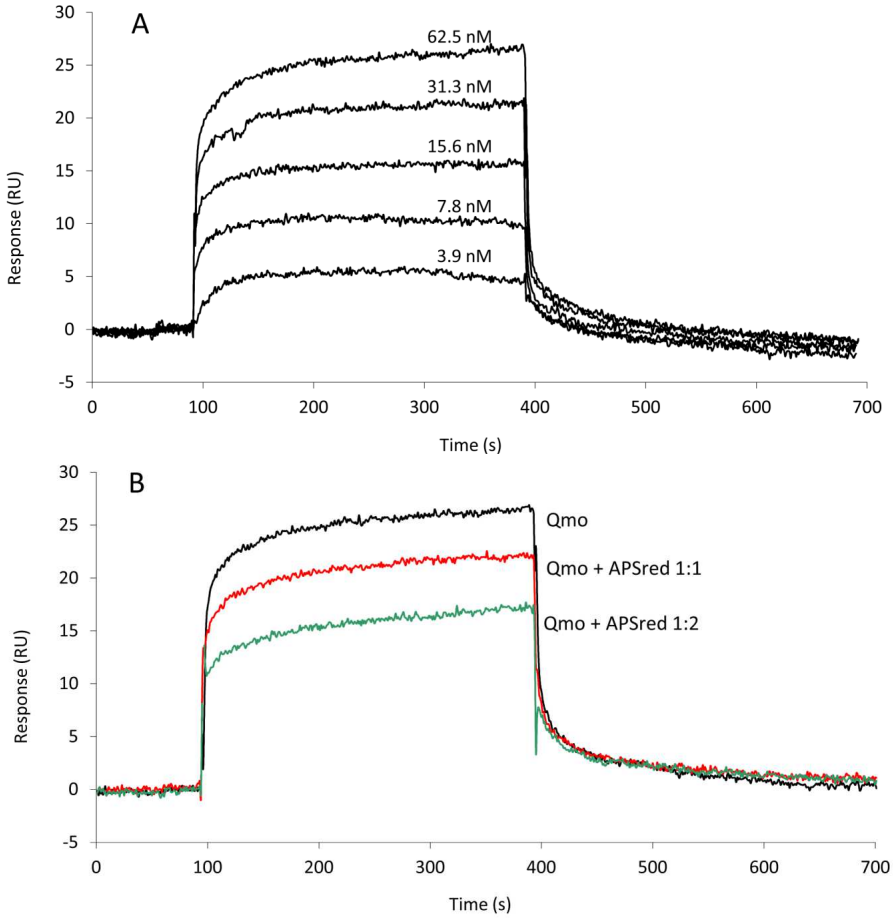


Figure 3.2 - Surface Plasmon Resonance analysis of the interaction between QmoABC (analyte) and immobilized AprBA (ligand). (A) Sensorgrams obtained from injection of serial dilutions of 62.5, 31.25, 15.6, 7.8, 3.9 nM QmoABC at 15 µl /min flow rate and 25 °C. (B) Competition experiment where QmoABC (62.5 nM) was mixed with 62.5 nM (1:1) or 125 nM (1:2) of AprBA before injection.

3.1.4.3 - Cross-linking Far-Western Blotting

Recently, a modification of the Far-Western protocol to include a cross-linking step was described, which allows for the detection of weak or transient interactions (Sato *et al.* 2011). Since a strong steady-state interaction was detected between QmoABC and AprBA, we used cross-

linking Far Western blot to try to elucidate which subunits are involved in this interaction. In this experiment, the QmoABC subunits were separated in a SDS-PAGE gel and blotted to a PVDF membrane. The membrane was incubated with AprBA, washed and EDC was then added to promote cross-linking to the retained protein, following which detection was performed by Western blot with Anti-AprBA antibodies (Figure 3.3). This showed a positive signal for the QmoA band and a weaker signal for the QmoC band. A shift in the molecular mass of the Qmo subunits is not expected to occur since they are already fixed in the membrane upon incubation with AprBA. No signals were detected when the experiment was run in the absence of cross-linker. In the reverse experiment where AprBA was run in the gel and the membrane was incubated with QmoABC, followed by cross-linking and detection with Anti-QmoABC antibodies, no signals could be detected. This indicates that in this case the denaturation of the AprBA subunits in SDS-PAGE prevents the interaction with QmoABC.

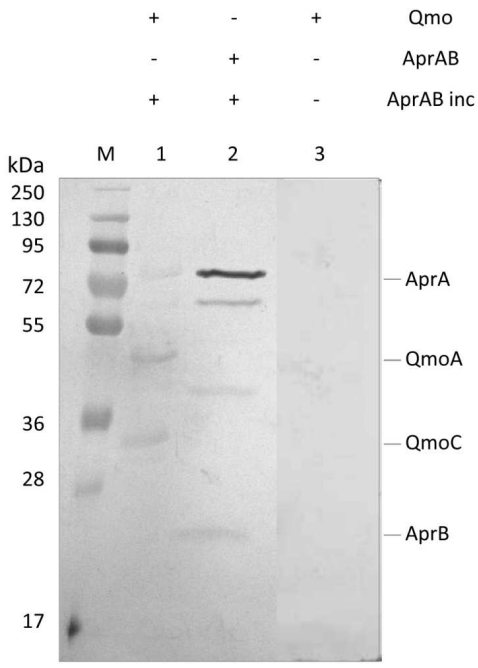


Figure 3.3 - Detection of interacting subunits by cross-linking Far Western Blot with Anti-AprBA antibodies. From left to right: M – Pre-Stained molecular mass markers; 1 - QmoABC in PVDF membrane was incubated with AprBA (AprBA inc), cross-linked with EDC and detected; 2- Positive control with AprBA in PVDF membrane; 3- Negative control with QmoABC in PVDF membrane not incubated with AprBA.

3.1.4.4 - PULL DOWN ASSAY

Since the QmoA protein is the subunit showing stronger interaction with AprBA, we set up an endogenous pull-down assay using single-epitope tag affinity purification based on tagged QmoA. No genetic tools are available for the organism *D. desulfuricans* ATCC 27774, but *D. vulgaris* Hildenborough can be genetically manipulated and extensive tools have been developed allowing chromosomal deletion and tagging of specific genes (Chhabra *et al.* 2011a; Chhabra *et al.* 2011b; Keller *et al.* 2011). A *D. vulgaris* Hildenborough mutant strain lacking the *qmoA* gene (IPAR02)

was produced by double homologous recombination, as previously described (Zane *et al.* 2010), and was complemented with plasmid pMOIP05 encoding *qmoA* with a Strep-TEV-FLAG (STF) tag to give strain IPAR03. This strain could grow on lactate/sulfate, in contrast to IPAR02 that only grew on lactate/sulfite, confirming that the complementation was successful. The QmoA protein was detected both in the membrane and in the soluble fraction of strain IPAR03 grown in lactate/sulfate. We took advantage of this fact to perform affinity tag purification of the soluble fraction using Strep-Tactin resin. The desthiobiotin elution fraction was analyzed by SDS-PAGE gel followed by Western blot with antibodies against *D. desulfuricans* AprBA (it was previously confirmed that these antibodies recognized the AprBA protein from *D. vulgaris*). A band for AprA was detected in the Western blot (Figure 3.4), confirming the ability of QmoA to interact and pull-down AprBA from the soluble fraction. In a parallel control experiment with wild-type cells of *D. vulgaris* Hildenborough no band was detected for AprBA.

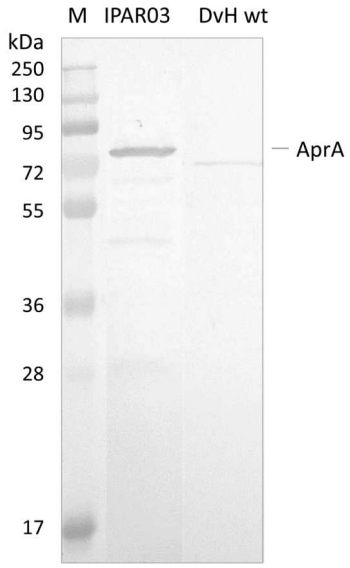


Figure 3.4 - Analysis of Pull down assay. Strep-tactin desthiobiotin elution products of soluble fraction from cells expressing STF-tagged QmoA (IPAR03) or wild type *D. vulgaris* (DvH wt, negative control) analysed by Western Blot with Anti-AprBA. M – Pre-Stained molecular mass markers.

3.1.4.5 - ELECTRON TRANSFER EXPERIMENTS

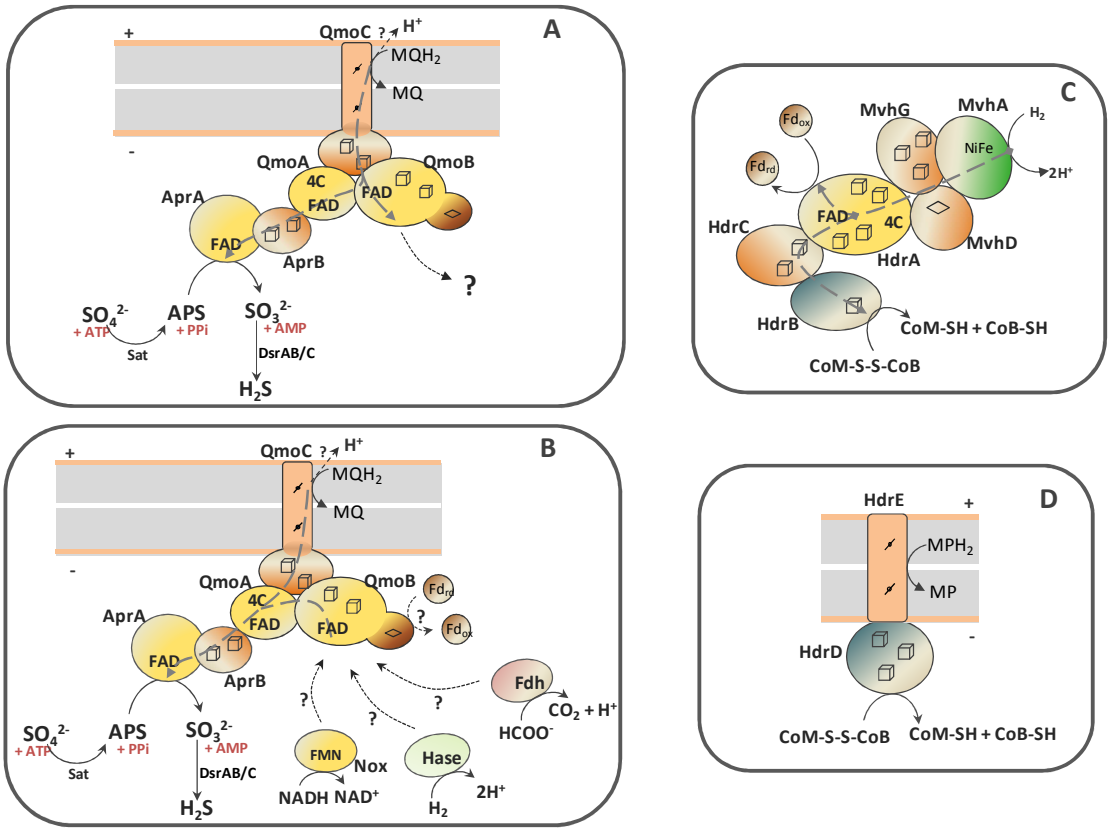
Since it was established that QmoABC can interact directly with AprBA we attempted again to observe electron transfer using anaerobically purified proteins. Previous experiments had been carried out with proteins purified aerobically (Pires *et al.* 2003), which could have suffered some damage to their iron-sulfur centers thus preventing electron transfer. We tested reduction of a menaquinone analogue (DMN) with sulfite (reverse reaction), or oxidation of quinol reduced QmoABC by APS (direct reaction) (Scheme 3.1). Despite a screening of different conditions, no evidence for electron transfer could be obtained.

3.1.5 - DISCUSSION

The AprBA APS reductase from SRP is a heterodimeric iron-sulfur flavoenzyme, which catalyzes the reversible reduction of APS to sulfite and AMP (Lampreia *et al.* 1994). It binds FAD, which is the site of APS reduction, and two [4Fe-4S] clusters that serve to transfer electrons from the protein surface to the catalytic site (Fritz *et al.* 2002a; Fritz *et al.* 2002b). Its physiological electron donor has not been unequivocally identified, but in many SRP and SOB the *aprBA* genes are part of a *sat-aprBA-qmoABC* gene cluster (Meyer and Kuever 2007; Frigaard and Dahl 2009; Gregersen *et al.* 2011; Pereira *et al.* 2011) which together with other indirect evidence (Pires *et al.* 2003; Haveman *et al.* 2004) led to the general conviction that QmoABC is the missing electron donor to AprBA, linking the quinone pool to sulfate reduction. The essential role of QmoABC in sulfate reduction has been recently established (Zane *et al.* 2010), but a direct connection between the two proteins has not been reported and direct electron transfer could not be observed (Pires *et al.* 2003). In some SOB lineages the *qmoABC* genes are absent and instead an *aprM* gene coding for a membrane protein is present (Hipp *et al.* 1997; Meyer and Kuever 2007; Frigaard and Dahl 2009), suggesting that AprM can substitute QmoABC in electron exchange between AprBA and the quinone pool. Homology modeling of AprBA from the different SOB lineages highlighted differences in the AprB structure that correlate with the presence of either the *qmo* or *aprM* genes, pointing to adaptation of the electron transfer protein AprB as a result of docking to either Qmo or AprM proteins (Meyer and Kuever 2008), and further substantiating a direct interaction.

In this work we report the first evidence that in SRP the QmoABC complex interacts directly with the APS reductase. This interaction could be detected by co-IP, and SPR showed that the two proteins are involved in a transient interaction that has a strong affinity (K_D= 90 ± 4 nM) in equilibrium conditions, and which has a fast dissociation rate. This property allowed the cross-linking of two proteins and detection by Far-Western blot, which revealed that the QmoA subunit, and to a less extent QmoC, is involved in the interaction. The reverse experiment gave no results, but in the case of AprBA it is known that AprA is the catalytic subunit and AprB the electron transfer subunit (Fritz *et al.* 2002a; Fritz *et al.* 2002b), so that interaction with the electron donor should involve AprB. Expression of a tagged version of QmoA in *D. vulgaris* Hildenborough, followed by affinity purification allowed the detection of co-eluting AprBA, further confirming a specific direct interaction between the two proteins in a physiological setting.

However, reduction of APS with a menaquinol analogue in the presence of QmoABC and AprBA could not be detected. The QmoABC subunits bind two hemes *b*, two FAD groups and several iron-sulfur centers, and are homologous to subunits of soluble (HdrABC) and membrane-bound (HdrED) heterodisulfide reductases from methanogens (Scheme 3.1) (Pires *et al.* 2003; Thauer *et al.* 2008; Kaster *et al.* 2011). QmoA and QmoB are both soluble iron-sulfur flavoproteins homologous to HdrA, the flavin-containing subunit of soluble HDRs.



Scheme 3.1 - Schematic representation of the QmoABC-AprBA interaction and the proposed involvement of third partners. A) In the hypothesis of an electron bifurcation process the putative electron acceptor of QmoB with a high redox potential is represented by a question mark. B) In the hypothesis of an electron confurcating mechanism several possible co-electron donors for the Qmo complex are considered: ferredoxin (Fd), hydrogenase (Hase), formate dehydrogenase (Fdh) or NADH dehydrogenase (Nox). The soluble HdrABC-MvhGAD complex (C) and the membrane-bound HdrED (D) of methanogens are shown for comparison. The grey dashed arrows represent electron bifurcation in A and C, or electron confurcation in B. The grey boxes represent the cytoplasmic membrane with + indicating the periplasm and – the cytoplasm.

The function of HdrA has not been completely established, but it has been proposed to be involved in flavin-based electron bifurcation carried out by a complex between HdrABC and the F_{420}-non-reducing MvhADG hydrogenase (Scheme 3.1C), which allows the coupling between the exergonic reduction of the CoM-S-S-CoB heterodisulfide by H_2 to the endergonic reduction of ferredoxin by H_2 (Thauer *et al.* 2008; Kaster *et al.* 2011). This bifurcation process is believed to involve the HdrA FAD cofactor, which transfers one electron to the heterodisulfide through HdrBC and another electron to ferredoxin. Such process may also occur with formate instead of H_2, with a formate dehydrogenase replacing the Mvh hydrogenase (Costa *et al.* 2010). QmoB includes also a domain similar to MvhD, the [2Fe-2S] subunit of the Mvh hydrogenase that is responsible for electron transfer to HdrABC (Scheme 3.1C) (Stojanowic *et al.* 2003). QmoC is a fusion protein that contains a cytochrome *b* transmembrane domain related to HdrE (Scheme 3.1D) and a hydrophilic iron–sulfur domain related to electron transfer subunit HdrC. Thus, QmoC fuses in a single protein the two subunits that in many trimeric respiratory oxidoreductases (composed of membrane subunit, electron transfer subunit and catalytic subunit) are responsible for electron exchange with the quinone pool and electron transfer to the catalytic subunit (Rothery *et al.* 2008; Simon *et al.* 2008). This leaves two subunits, QmoA and QmoB, with an unknown function and which will likely interact with other physiological partners. QmoA is shown here to interact with AprBA, but the function of QmoB remains enigmatic. Its similarity to HdrA and MvhD suggests the involvement of a third physiological partner for the Qmo complex. We must also consider that

menaquinol ($E^{O'}$ – 75 mV) cannot serve as sole electron donor to reduce APS ($E^{O'}$ APS/SO_3^{2-} = – 60 mV) due to the small difference in redox potentials, and to the fact that the membrane potential (~150 mV) has to be overcome when transferring electrons from the quinone binding site in QmoC (likely situated towards the periplasmic side of the membrane) to AprBA in the cytoplasm. Thus, the reduction of APS by menaquinol has to be driven by coupling it to a second more favorable reaction. The idea that an electron bifurcation or confurcation mechanism, originally proposed by Buckel and coworkers (Herrmann *et al.* 2008), could be operating in the reduction of APS then appears as a very attractive and plausible hypothesis. Two possibilities can be envisioned: In the first one (Scheme 3.1A) the QmoB subunit reduced by menaquinol could bifurcate electrons to QmoA/AprBA and to a second electron acceptor with a high redox potential. The energetically favorable reduction of such electron acceptor by menaquinol could drive the unfavorable reduction of APS by menaquinol. The only problem with this hypothesis is that we cannot identify a candidate in SRB with a high enough reduction potential to drive this reaction.

The second possibility, that we favor, is to consider a reverse electron bifurcation mechanism, which has been referred to as electron confurcation. In such a process menaquinol and a cytoplasmic reductant of low redox potential could both serve as electron donors to the Qmo complex, which would confurcate electrons to the APS reductase (Scheme 3.1B). The favorable reduction of APS by this low potential electron donor would drive the unfavorable reduction of APS by menaquinol. The process of bifurcation/confurcation requires the

presence of a two-electron center, such as a flavin, as the coupling site. In Qmo there are two FAD cofactors that can perform this process. According to the idea of crossed potentials at the flavin proposed by Nitschke and Russell (Nitschke *et al.* 2011), the reduction of FAD at QmoA or QmoB by the low potential electron donor could generate a "hot" flavosemiquinone with a high redox potential that would then be a favorable electron acceptor for a second electron coming from menaquinol, and in practice "pulling" this electron from the quinone. Electron confurcation has been reported in the reduction of $NADP^+$ with both reduced ferredoxin and NADH by *Clostridium kluyveri* NfnAB (Wang *et al.*, 2010), and also in a multimeric soluble [FeFe] hydrogenase from *Thermotoga maritima*, which uses both NADH and reduced ferredoxin to produce H_2 (Schut *et al.* 2009). This process has also been implicated in the energy metabolism of syntrophic organisms (Müller *et al.* 2010; Sieber *et al.* 2010).

Several coupling partners for Qmo can be considered in the confurcation hypothesis. The first is a hydrogenase or a formate dehydrogenase by analogy to what happens with HdrABC of methanogens (Costa *et al.* 2010; Kaster *et al.* 2011). An analysis of SRP genomes showed that a cytoplasmic version of either one of the two enzymes is always present (Pereira *et al.* 2011), except in *C. maquilingensis* where the *qmoABC* genes are also absent. In several organisms an MvhADG homologue is present, which in the archaeal and in some bacterial organisms is part of an *mvhADG-hdrABC* gene cluster, suggesting this was acquired by lateral gene transfer from methanogenic organisms. In other bacteria the *mvhADG* genes are isolated, which may indicate subsequent loss of the

hdrABC genes. In *Desulfovibrio* organisms no *mvhADG* genes are present, but genes coding for a membrane-associated hydrogenase (Ech or Coo) or a soluble [FeFe] hydrogenase are detected. The second possible partner for QmoB is a ferredoxin, also by analogy to HdrA. Ferredoxins are present in the genomes of all SRP, often in multiple copies (Pereira *et al.* 2011). Several proteins in SRP are known to reduce ferredoxin, including hydrogenases and formate dehydrogenases, pyruvate:ferredoxin oxidoreductase and the Rnf complex, which is also present in several *Desulfovibrio* sp. (Pereira *et al.* 2011). Finally, a third possible partner of QmoB is the mononuclear NADH oxidoreductase, Nox, which has been reported to reduce AprBA (Chen *et al.* 1994). Nox homologues (DVU3212 in *D. vulgaris* Hildenborough) are also present in the 25 genomes of SRP analyzed, except *Thermodesulfovibrio yellowstonii*. Recently, a study of protein-protein interactions failed to detect a link between Nox and energy metabolism proteins (Chhabra *et al.* 2011b), but such a negative result is not entirely conclusive due to the possibility of transient interactions not being detected in the conditions used. In these hypotheses H_2 ($E^{o'}$ -414 mV), formate ($E^{o'}$ -430 mV), NADH ($E^{o'}$ -320 mV) or ferredoxin ($E^{o'}$ ~ -400 mV), would all be favorable reductants for APS ($E^{o'}$ APS/SO_3^{2-} = -60 mV). It is conceivable that more than one of these compounds may be used depending on the metabolic conditions, as observed for HdrABC (Costa *et al.* 2010), which could explain why no genes for interacting partners are co-localized with the *sat-aprBA-qmoABC* gene cluster. Any of these reductants could serve as a sole electron donor for the reduction of APS on its own, but in such situation the cells would get no energy benefit from this step. Coupling

of APS reduction with oxidation of the menaquinone pool allows for energy conservation, considering that the oxidation of menaquinol by QmoC occurs at the periplasmic side of the membrane, with release of protons to the periplasm. In conclusion, the confurcation mechanism proposed here effectively allows the coupling of sulfate reduction with chemiosmotic energy conservation, a process long known to occur in SRP, but for which the molecular basis has been hard to identify. Clearly, further experiments will be required to test this hypothesis.

3.1.6 - ACKNOWLEDGEMENTS

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CHAPTER 3

SECTION 3.2

REVERSE ELECTRON BIFURCATION: A LINK BETWEEN THE MENAQUINONE POOL AND SULFATE REDUCTION?

3.2.1 - SUMMARY

The dissimilatory sulfate reduction pathway is an intracellular process in which sulfate is actively transported inside the cell and reduced to the final product sulfide, with APS and sulfite as intermediate metabolites. Although the enzymes involved in the process are well characterized, the mechanism of energy conservation is not completely understood. This work focused in the electron transfer pathway for APS reduction, involving the QmoABC membrane complex and AprBA. The QmoABC complex interacts *in vitro* and *in vivo* with AprBA (Ramos *et al.* 2012), and it is essential for sulfate reduction (Zane *et al.* 2010). However, no direct electron transfer was ever observed between the two proteins, and a mechanism of electron confurcation was proposed to operate in APS reduction with Qmo and AprBA (Ramos *et al.* 2012). In this work we performed several electron transfer experiments to study the possible electron transfer between the quinone pool and APS reduction. The assays included direct electron transfer and electron bifurcation or confurcation experiments. An HPLC analysis was adopted for sulfite quantification, which allowed detection of sulfite production with QmoABC, menadiol, AprBA, reduced ferredoxin [using pyruvate, coenzyme A (CoA) and pyruvate: ferrodoxin oxidoreductase (POR)] and APS, but a similar result was obtained when only the AprBA and POR system (POR, pyruvate and Coenzyme A) were present. We discuss future experiments with Qmo reconstituted in liposomes to study the mechanism of electron transfer and possible energy conservation in sulfate respiration.

3.2.2 - INTRODUCTION

The isolation and characterization of the QmoABC complex by (Pires *et al.* 2003) brought to attention several features of this membrane protein which suggested an involvement in the sulfate reduction pathway. The first evidence was that the *qmo* genes cluster with *aprBA* genes in many SRB and SOB, suggesting a direct link between the two proteins. Later, strong evidence was obtained indicating that Qmo complex is required for sulfate reduction in SRB (Zane *et al.* 2010) or sulfite oxidation in green sulfur bacteria (Rodriguez *et al.* 2011). Additionally, in the previous section, protein-protein interaction studies demonstrated that QmoA interacts directly with AprBA (Ramos *et al.* 2012), establishing for the first time a physical connection between the two proteins. However, electron transfer between menaquinol analogs and APS through QmoABC and AprBA could never be detected.

A reverse electron bifurcation, i.e., electron confurcation was proposed with the involvement of a third partner, by coupling APS reduction with oxidation of both menaquinol and a low redox potential electron donor (possibly ferredoxin). The proposal takes into account the small redox potential difference between menaquinol ($E^{0'}$ = – 75 mV) and APS ($E^{0'}APS/SO_3^{2-}$ = – 60 mV), and also the fact that the membrane potential (~ 150 mV) has to be overcome when electrons are transferred from the quinone binding site in QmoC to AprBA in the cytoplasm.

In this work we performed several *in vitro* experiments to try to find evidence of direct electron transfer or flavin-based electron bifurcation/confurcation in the sulfate reduction pathway involving QmoABC and APS reductase complexes.

3.2.3 - MATERIAL AND METHODS

3.2.3.1 - BIOCHEMICALS

Adenosine 5'-monophosphate (AMP), sodium sulfite , methyl-viologen (MV), metallic zinc, NADH, flavin adenine dinucleotide (FAD), dithiothreitol (DTT), Menadione, adenosine 5'-phosphosulfate (APS), coenzyme A (CoA), potassium ferricyanide ($K_3[Fe(CN)_6]$), ferrocenium hexafluorophosphate and iodonitrosotetrazolium chloride (INT) were from Sigma-Aldrich®; Sodium pyruvate was from Carl Roth® (Germany). The menaquinone analog 2,3-dimethyl-1,4-naphthoquinone (DMN) was synthesized according to (Kruber 1929), and was used in the oxidized form or was reduced with metallic zinc in an ethanolic solution containing 0.2 M HCl. Quantification was performed by UV-Visible spectroscopy (ε_{DMN} at 270 nm = 16 $mM^{-1}cm^{-1}$) and considering that DMN absorbs strongly at 270 nm, while the reduced form ($DMNH_2$) has low absorbance at this wavelength. Menadiol was obtained by reduction of menadione with sodium dithionite as described in (Fieser 1940).

3.2.3.2 - PREPARATION OF CELL EXTRACTS AND PROTEINS PURIFICATION

Cell extracts from *D. desulfuricans* ATCC 27774 were prepared by suspending 2 g of cells grown according to (Pires *et al.* 2003) in 10 mM Tris-HCl (pH 7.6). Cells were broken in a French Press and centrifuged for 20 min at 5,136 × *g*.

D. desulfuricans membrane extracts were prepared by suspending 11 g of cells followed by disruption under N_2 (95%), and centrifugation for 90 min at 137,000 × *g*. Membranes were suspended in 20 mM phosphate

buffer (pH 7) containing 20 mM $MgSO_4$, 500 mM sucrose, 5 mM DTT and 1 µg/ml (w/v) resazurin (Welte and Deppenmeier 2011a). The membranes were again centrifuged and suspended in the same buffer (washed membranes). The protein concentration in extracts was determined by the Bradford method (Sigma) with bovine gamma globulin as the standard (NZYTech).

AprBA and QmoABC were purified as described in Section 3.1; ferredoxin (Fd) was purified from *D. vulgaris* Hildenborough (Ogata *et al.* 1988) or from *C. tetanomorphum* (DSM 526) (Palchowdhury *et al.* 2013); pyruvate:ferredoxin oxidoreductase (POR) was purified from *D. africanus* (Pieulle *et al.* 1995). In some experiments, anaerobic *D. desulfuricans* fractions containing Fd and NADH oxidoreductase (Nox) were used. These preparations obtained during AprBA purification, eluted at higher ionic strength (more than 350 mM NaCl) and were identified by the visible spectrum in the case of ferredoxin (absorbance at 410 nm) and, NADH oxidization activity and co-elution with desulfoviridin, in the case of Nox.

3.2.3.3 - BIOCHEMICAL ANALYSIS

QmoABC and AprBA integrity was routinely checked by cofactor quantification, UV-visible spectroscopy, SDS-PAGE and, in the case of AprBA, enzymatic activity. Non heme iron content was determined by the 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) method (Fischer and Price 1964); Qmo heme *b* content was determined by the pyridine hemochrome derivative as described in (Berry and Trumpower 1987); Flavins were extracted with trichloroacetic acid (Susin *et al.* 1993), and

their content determined by fluorescence spectroscopy using free FAD as standard.

3.2.3.4 - ENZYME ACTIVITY ASSAYS

AprBA activity was performed as described in Section 3.1, either as APS formation monitoring $K_3[Fe(CN)_6]$ reduction at 420 nm ($\varepsilon_{potassium\ ferricyanide}$ at 420 nm = 1.05 $mM^{-1}\ cm^{-1}$) or as AMP and sulfite formation monitoring MV oxidation at 732 nm (ε_{MV} at 732 nm = 3.15 $mM^{-1}\ cm^{-1}$) (Fritz *et al.* 2002a). POR activity was monitored by MV reduction at 604 nm (ε_{MV} at 604 nm = 13.9 $mM^{-1}\ cm^{-1}$) in 50 mM Tris-HCl (pH 8.5) with 1mM MV, 0.1 mM CoA, 10 mM DTT and 10 mM pyruvate. NADH activity was also determined following $K_3[Fe(CN)_6]$ reduction at 420 nm in 50 mM Tris-HCl (pH 7.6), 1.2 mM $K_3[Fe(CN)_6]$ and 50 µM NADH.

3.2.3.5 - SPECTROSCOPIC TECHNIQUES

UV-visible absorption spectra and spectrophotometer assays were performed inside an anaerobic chamber (95% N_2, 5% H_2 atmosphere) with a Shimadzu UV-1203 or Shimadzu UV-1800 spectrophotometers. Aerobic measurements were performed with a Shimadzu UV-1603 spectrophotometer. The experiments were performed using quartz cuvettes (Hellma) equipped with a magnetic stirrer. Fluorescence spectra were recorded on a Varian Cary Eclipse spectrofluorimeter, using fluorescence quartz cuvettes (Excallibur instruments).

3.2.3.6 - ELECTRON TRANSFER ASSAYS

The direct and bifurcation/confurcation electron transfer experiments were performed in 10 mM Phosphate buffer (pH 7) and 0.0125% DDM (w/v) (Buffer A) except in the following situations: in the direct electron transfer assays with $DMNH_2$ we used Buffer A with 2.5 mM EDTA; the confurcation experiments with NADH and *D. desulfuricans* cell extracts were performed in 50 mM Tris-HCl (pH 7.6); in the assays with *D. desulfuricans* membranes and H_2, Buffer A was saturated with H_2 by flushing the serum bottle with H_2 gas; and the HPLC assays were performed in Buffer A supplemented with 5 µM FAD. The experiments were performed anaerobically at room temperature with the exception of the reactions for the HPLC measurements that were incubated at 37 ^{o}C. AprBA reduction was followed at 390 nm and Qmo heme *b* oxidation at 424 and 556 nm. Menadiol oxidation was followed at 320 nm and DMN reduction at 350 nm. NADH oxidation was measured at 340 nm. Clostridial Fd oxidation was monitored at 390 nm.

To investigate Qmo quinol oxidase activity, different artificial electron acceptors were tested. Potassium ferricyanide, ferrocenium hexafluorophosphate and INT were tested with Qmo (0.1 µM) in the presence of menadiol and $DMNH_2$. The experiments were conducted anaerobically in Buffer A; Reduction of 1 mM $K_3[Fe(CN)_6]$ was tested at 420 nm with 200 µM $DMNH_2$ and different concentrations of menadiol (10, 25, 50 and 100 µM); Reduction of 200 µM ferrocenium hexafluorophosphate (one electron acceptor) was measured at 300 nm with addition of menadiol; and reduction of 50 or 100 µM INT was

measured at 492 nm with $DMNH_2$ or menadiol. In control reactions Qmo was absent from the reaction.

3.2.3.7 - COLORIMETRIC DETERMINATION OF SULFITE

Colorimetric quantification of sulfite was adapted from the assay for sulfur dioxide determination with fuchsin reagent (Grant 1947). The method is sensitive over a range of 0 to 40 nmol of sulfite. Sodium sulfite standards were prepared to cover a series of concentrations between 0 and 250 µM. A solution of pararosaniline-HCl (Sigma) 0.04% (w/v) in H_2SO_4 10% (v/v) (Fuchsin reagent), and fresh formaldehyde (Sigma) 3.7% (v/v) were used for the assays. After each experiment, the sulfite-containing sample was diluted in anoxic MilliQ water (final volume 800 µl) and incubated with Fuchsin reagent (100 µL) for 9 min in the dark. Formaldehyde solution (100 µl) was then added, mixed and again the reaction was incubated for 9 min in the dark. The tubes were then removed from the dark and 750 µl of the reaction was transferred into a cuvette containing 750 µl of water, homogenized and the absorbance was measured at 570 nm, blanked with water. Sulfite concentrations were calculated based on a calibration curve and discounting the absorbance values from the control reactions.

3.2.3.8 - HPLC DETERMINATION OF SULFITE

An HPLC method was used to quantify sulfite based on the use of monobromobimane (mBBr), a fluorescent reagent that reacts with thiols and reduced sulfur compounds (Gru *et al.* 1998). A 50 mM mBBr stock solution was prepared in HPLC ultra pure acetonitrile. For derivatization

10 µl of sample was incubated with 10 mM mBBr in 20 mM HEPES (pH 8) buffer in the dark for 10 min. The reaction was stopped by addition of 50 mM methane sulfonic acid. For the HPLC analysis, samples were diluted 10x in 10 mM methane sulfonic acid. Sulfite analysis was performed on a HPLC (Waters Alliance 2695) equipped with a fluorescence detector (Waters 486) with excitation and emission wavelengths of 380 nm and 480 nm, respectively. A reverse-phase Ultrasphere C18 Beckman Coulter column (4.6 x 250 mm; 5 µm) was used at 35^{o}C. The gradient was performed with 0.25% (v/v) acetic acid pH 4 in MilliQ water (A) and 100% Methanol (B). The elution protocol was as follows: 0 min 80% A, 20% B; 13 min 50% A, 50% B; 16 min 48% A, 52% B; 20 min 0% A, 100% B; 26 min 80% A, 20% B. The flow-rate of the mobile phase was 1.20 mL/ min, while the injection volume was 50 µl. The sulfite retention time was about 3.44 min.

3.2.4 - RESULTS

In the previous section protein-protein interaction studies revealed that QmoABC interacts directly with AprBA in *Desulfovibrio* spp. The interaction was characterized and it was concluded that it is strong but with a transient character, as expected for proteins involved in electron transfer (Bashir *et al.* 2011; Martinez-Fabregas *et al.* 2011). However, direct electron transfer between the two proteins was not observed and the idea that an additional partner may be required was proposed (Ramos *et al.* 2012). To support this idea is the fact that QmoA and QmoB share homology to HdrA, the heterodissulfide reductase subunit proposed to perform flavin-based electron bifurcation in methanogens

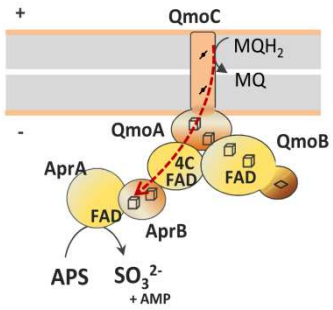
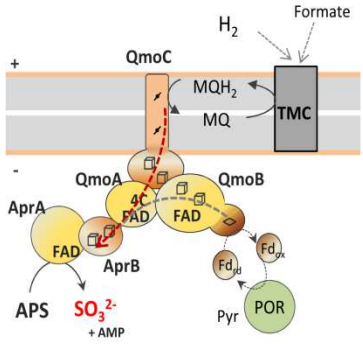
(Buckel and Thauer 2013; Costa *et al.* 2013), suggesting the operation of a similar mechanism in sulfate reduction. A reverse electron bifurcation, or electron confurcation mechanism was proposed to operate in APS reduction coupling it to the oxidation of the menaquinone pool (Ramos *et al.* 2012).

3.2.4.1 - DIRECT ELECTRON TRANSFER

Initially we repeated experiments by Pires *et al.* (Pires *et al.* 2003) and tested direct AprBA (2.7 μM) reduction (at 390 nm), upon addition of $DMNH_2$ (140 μM) and QmoABC (1.3 μM); or, in alternative, oxidation of Qmo (3.8 μM) hemes (at 424 nm) pre-reduced with $DMNH_2$ (276 μM), upon addition of AprBA (2.5 μM). In other experiments several concentrations of menadiol (25, 50, 100, 300, 930 μM) were tested to reduce Qmo (0.3 μM) hemes, and heme oxidation was followed upon addition of AprBA (0.1 μM) and APS (30, 60 and 120 μM). As before, no evidence for electron transfer between Qmo and AprBA was observed.

In another experiment menadiol (25 μM) oxidation was followed at 320 nm with Qmo (0.3 μM), APS reductase (0.1 μM) and APS (30 μM), but again no evidence for direct electron transfer was obtained. These spectrophotometric experiments include several different components with similar absorbance spectra (Figure 3.5), which can be problematic. Thus, other options were explored, namely the detection of sulfite as reaction product first with the Fuchsin reagent and later by HPLC.

Table 3.3 - Reaction components of direct electron transfer experiments. MQ, menaquinol; TMC, generic transmembrane complex.

	Scheme of the reaction	Electron donor	Electron acceptor	Method
1		$DMNH_2$	AprBA	AprBA red. (390 nm)
		Qmo reduced by $DMNH_2$ or menadiol	AprBA APS	Qmo ox. (424 nm); Sulfite (Fuschin or HPLC)
		Menadiol	APS	Menadiol ox. (320 nm)
2		Formate; H_2	APS	Sulfite (Fuschin)

For sulfite quantification with Fuchsin, the reaction mixture (0.3 µM Qmo, 25 µM menadiol, 0.1 µM AprBA and 30 µM APS) was incubated for 1 h at room temperature, using as controls the mixture lacking APS or AprBA. As such, we used an HPLC method to quantify the sulfite based on the use of monobromobimane (mBBr), a fluorescent reagent that reacts with thiols and reduced sulfur compounds (Gru *et al.* 1998). To test the method we first used it to measure AprBA activity with methyl viologen, and the sulfite produced after 2 min (12 µM) agreed with the

APS rate consumption (6 µmol APS reduced $min^{-1}mg^{-1}$AprBA). We tested the same reaction conditions as for the fuchsin detection (0.3 µM Qmo, 0.1 µM AprBA and 30 µM APS), but with a different menadiol concentration (750 µM) and 37 ^{o}C as temperature of incubation. Control reactions were performed by omitting individual components. No sulfite was detected in the conditions tested for direct electron transfer.

We tested also artificial electron acceptors to measure quinol oxidation activity of Qmo. We tested potassium ferricyanide ($E^{0'}$= + 430 mV, Sigma®), ferrocenium hexafluorophosphate ($E^{0'}$ = + 380 mV, (Li *et al.* 2008) and INT ($E^{0'}$ = – 90 mV; (Smith and McFeters 1997)). The assay evaluated the capacity of each oxidant to receive electrons from Qmo reduced with menaquinol analogs, and as a control the Qmo was excluded from the system. Unfortunately, direct electron transfer between the quinol analogues and the artificial electron acceptors was observed, thus these electron acceptors are not suitable to measure quinol oxidation activity.

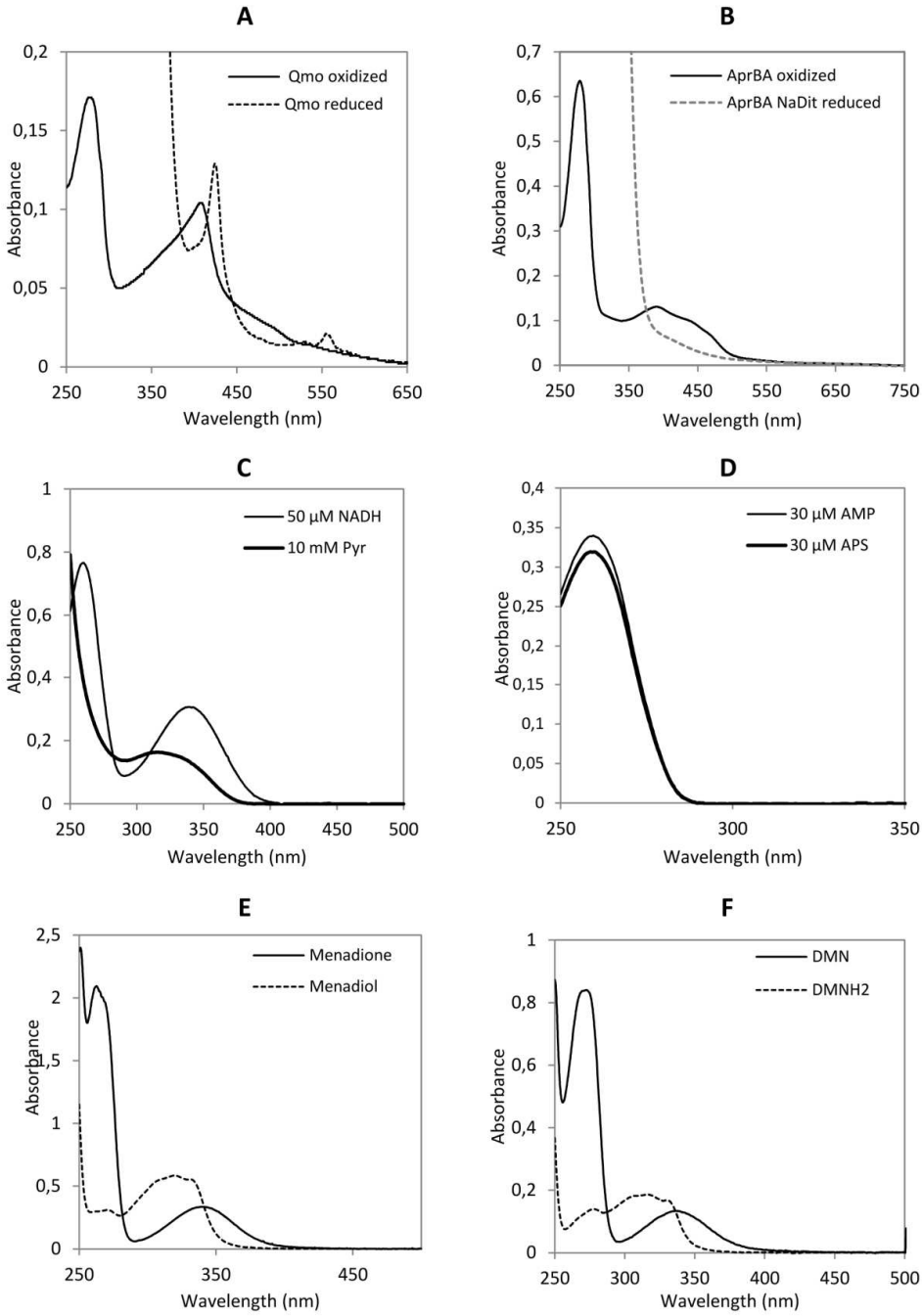


Figure 3. 5 - Spectroscopic characteristics of diferent components used in the electron transfer reactions. (A) QmoABC oxidized and reduced with sodium dithionite; (B) AprBA oxidized and sodium dithionite reduced; (C) NADH and pyruvate; (D) AMP and APS; (E) menadione and menadiol; (F) DMN and $DMNH_2$.

Finally, *D. desulfuricans* membranes were tested in the presence of formate and H_2 as possible electron donors (Table 3.3, reaction 2). As a control we used the reaction mixture lacking APS. Sulfite was determined with Fuchsin after 2h incubation with AprBA (0.1 µM) and APS (30 µM), or after incubation with AprBA, APS, pyruvate (5 mM), POR (2.3 nM) and *D. desulfuricans* Fd (2 µM) (Table 3.3). Some sulfite production was observed, but the results were not consistent (Table 3.4). This may be due to the fact that the sulfite produced may be consumed by some DsrAB that remains associated to the membrane.

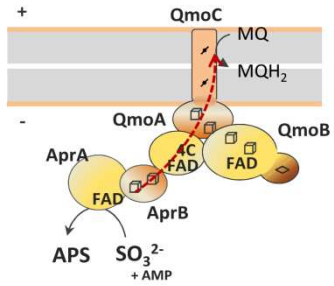
Table 3. 4 - Sulfite produced by membrane extracts of *D. desulfuricans* determined by colorimetric fuchsin assay.

Membranes (µg)	$[SO_3]$ (µM)	Electron donor
50	5.8	Formate
100	4.9	Formate
	3.4	H_2
	18.4	Formate
200	18.4	H_2
	9	H_2, pyr, POR, Fd

3.2.4.2 - REVERSE DIRECT ELECTRON TRANSFER

A direct electron transfer, based on AprBA reverse activity, was performed following the reduction of the menaquinone analogue DMN at 350 nm. In the AprBA reverse activity, APS is formed from sulfite and AMP. We tested DMN (500 µM) reduction with 2 mM Na_2SO_3, 2 mM AMP and 0.5 µM of QmoABC after addition of 1.2 µM AprBA. No evidence of DMN reduction was observed.

Table 3.5 - Reaction components of reverse direct electron transfer experiments.

Scheme of the reaction	Electron donor	Electron acceptor	Method
	AprBA (SO_3^{2-} + AMP)	DMN	DMN red (350 nm)

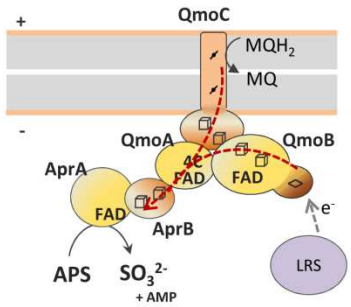
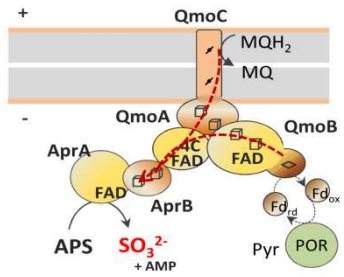
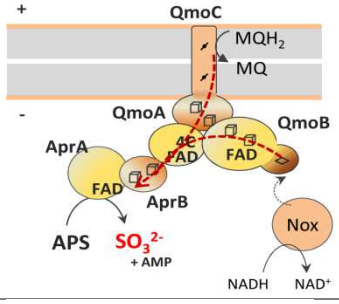
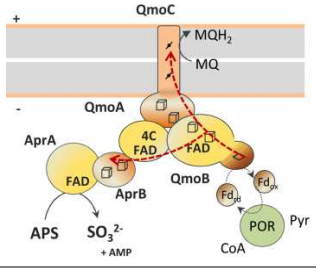
3.2.4.3 – BIFURCATION/CONFURCATION ELECTRON TRANSFER

Since we could not find evidence for direct electron transfer we started to investigate the possibility of confurcation or bifurcation reactions. Initially, as a control we used *D. desulfuricans* cell extracts and NADH (50 µM) as the first possible soluble electron donor following the oxidation at 340 nm upon addition of APS (30 and 60 µM) (Table 3.6, reaction 1). NADH (50 µM) oxidation was also tested in the presence of Qmo (0.3 µM) reduced with menadiol (25 µM), AprBA (0.1 µM) and APS (30 µM). Besides following NADH oxidation we measured also Qmo heme *b* oxidation. Cell extracts were also used to assess menadiol (93 µM) oxidation at 320 nm in the presence of APS (30 µM) and 2.5 mM pyruvate to promote Fd reduction.

We then tested the pure proteins in two types of confurcation experiments. In one type we measured menadiol oxidation (50 µM; 320 nm) in the presence of Qmo (0.3 µM), AprBA (0.1 µM), POR (2.3 nM),

pyruvate (500 µM), partially purified *D. desulfuricans* Fd (2 µM) and APS (50 µM) – Table 3.6, reaction 2. In the second type, NADH (50 µM) and quinol oxidation were measured (300 nm) in the presence of Qmo (0.3 µM), menadiol (50 µM), AprBA (0.1 µM), NADH (50 µM), partially purified Nox (~0.1 µM) and APS (50 µM) – Table 3.6, reaction 3. The reactions mixtures were incubated for 2 h and sulfite was determined by Fuchsin, using as controls the reaction mixture without APS. In all experiments no evidence of sulfite formation was observed. The results from the colorimetric sulfite quantification were not reproducible and sometimes the controls presented higher absorbance than the reactions. We found that menadiol interfered in the method and a reliable result was never obtained. For this reason this method was abandoned.

Table 3.6 - Reaction components of confurcation and bifurcation reactions tested. MQ – menaquinone; LRS - low redox potential species; Pyr – pyruvate.

	Scheme of the reaction	Electron donor	Electron acceptor	Method
1		NADH	(Cell extracts) APS	NADH ox (340 nm)
		NADH and menadiol	(Pure proteins) APS	NADH ox (340 nm) and Qmo heme ox (424 nm)
2		Menadiol and POR system-reduced Fd	APS	Menadiol ox (320 nm) Sulfite (Fuchsin or HPLC)
3		Menadiol and NADH	APS	Menadiol and NADH ox (300 nm) Sulfite (Fuchsin)
4		POR system - reduced Fd	APS and menadione	Sulfite (HPLC)

The HPLC method for sulfite quantification was then used to evaluate possible electron transfer by confurcation/bifurcation using the pure proteins. In the confurcation experiment we tested Qmo (1 μM) reduced by menadiol (750 μM), together with clostridial Fd (10 μM) reduced by the POR system (10 mM pyruvate, 100 μM CoA, 0.54 nM POR), in the presence of AprBA (0.1 μM) and APS (50 μM). In the bifurcation experiment we tested if electrons from reduced Fd bifurcate to menadione and AprBA, thus the reaction mixture contained menadione (330 μM) instead of menadiol. The reactions were incubated for 80 minutes at 37 ^{o}C, with samples removed after 0, 5, 10, 20, 40 and 80 minutes, which were derivatized with mBBr. Several controls were performed by omission of individual components in the reaction.

The HPLC analyses did not detected sulfite in the bifurcation reaction, but in the bifurcation control reaction without menadione, traces of sulfite were detected. The results obtained for the confurcation reaction are presented in Figure 3.6 , and as we can see, sulfite is detected in three particular experiments: (1) in the presence of Qmo, POR system and Fd, (2) with Qmo and POR system, and (3) only with the POR system. The assays were repeated several times giving always similar results. So, POR alone is capable of transferring electrons to AprBA to reduce APS.

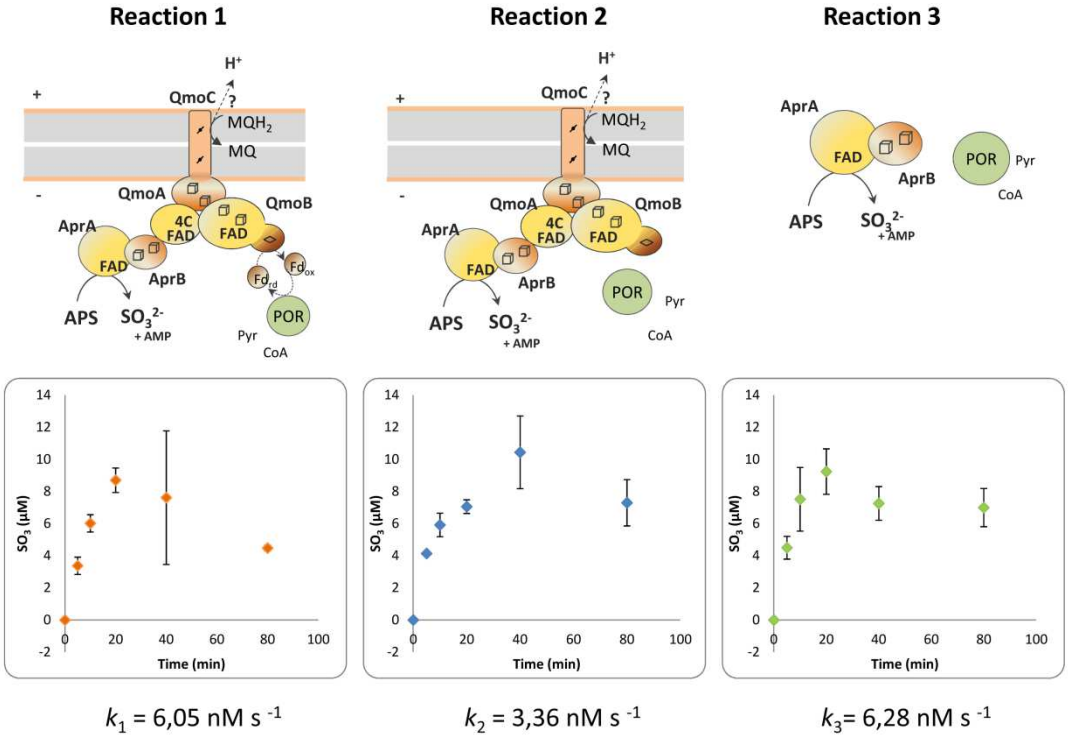


Figure 3.6 - Top: Schematic representation of the reactions components of three experiments used to measure sulfite over time by HPLC. Reaction 1- "confurcation" with Qmo, Fd and POR system; Reaction 2 – "confurcation" with Qmo and POR system; Reaction 3 - direct reaction with AprBA and POR system. Bottom: graphical representation of sulfite (µM) generated over time of each reaction with the respective rate constants (*k*, nM min^{-1}).

The electron confurcation and bifurcation experiments were also monitored by UV-vis spectroscopy in parallel with the HPLC measurements. Despite evidence of sulfite formation by HPLC, by UV-vis we could not find evidence for menadiol oxidation (320 nm), heme *b* oxidation (424 nm) or Fd oxidation (390 nm). In HPLC measurements sulfite formation is observed during the first 20 to 30 min, so changes in

the UV-vis spectra would be expected in the same time range, which were not visible.

3.2.5 - DISCUSSION

The mechanism of electron transfer and possible energy conservation in sulfate reduction is beginning to take shape, due to genomic, transcriptomic, proteomic and biochemical studies that have emerged in the last years (Muyzer and Stams 2008; Pereira 2008; Walker *et al.* 2009; Venceslau *et al.* 2010; Keller and Wall 2011; Pereira *et al.* 2011; Grein *et al.* 2013). Sulfate respiration is associated with oxidative phosphorylation, but the process of electron transfer is still to be fully understood (Pereira *et al.* 2011). The QmoABC and DsrMKJOP are strictly conserved in SRO and proposed to be the physiological partners of the two terminal reductases AprBA and DsrAB (Grein *et al.* 2013), linking the membrane quinone pool to sulfate respiration.

This work focused on the QmoABC complex and the possible electron bifurcation/confurcation mechanism involved in electron transfer from the menaquinone pool to APS reduction in the cytoplasm. When the QmoABC complex was isolated and characterized (Pires *et al.* 2003), the finding that *qmo* genes cluster with *aprBA* genes in many SRO (and also SOB), suggested the involvement of Qmo in electron transfer from the quinone pool to AprBA. However, electron transfer between this membrane complex and APS reductase was tested and not observed, raising doubts about their connection. The essential role of QmoABC in sulfate reduction was later determined by the work of (Zane *et al.* 2010) where a mutant lacking the *qmo* genes was not able to grow on sulfate,

but grew well on sulfite. The work described in the previous section proved for the first time that there is a direct physical interaction between QmoABC and AprBA *in vitro* and also *in vivo*. The interaction was characterized as having a transient character, typical of proteins involved in electron transfer, and the QmoA subunit was shown to be involved in the interaction with AprBA (Ramos *et al.* 2012). The work of (Krumholz *et al.* 2013) also confirmed this interaction in *D. alaskensis* G20 by analysis of membrane protein complexes.

Since no electron transfer was observed between Qmo and Apr the hypothesis of a mechanism of electron confurcation was proposed (Ramos *et al.* 2012). The proposal was based on the fact that the subunits QmoA and QmoB have homology to HdrA, the flavin containing subunit of HdrABC in methanogens that is proposed to perform flavin-based electron bifurcation (Thauer *et al.* 2008). In methanogens lacking cytochromes, a soluble complex formed between HdrABC and MvhADG is responsible for coupling the endergonic reduction of Fd by H_2 with the exergonic reduction of the CoM-S-S-CoB heterodisulfide by H_2 (Thauer *et al.* 2008; Kaster *et al.* 2011). In addition, the proposal also took into account the redox potentials involved in the reaction. The QmoC hemes (+75 mV and –20 mV) are reduced by menaquinol analogs, and are probably involved in electron transfer from menaquinol to the cytoplasmic electron acceptor (Pires *et al.* 2003). However, there is a small difference in redox potential between menaquinol (–75 mV) and APS ($E^{0'}$ APS/SO_3^{2-} = –60 mV). Furthermore, the membrane potential (~150 mV) has to be overcome when transferring electrons from the QmoC to AprBA in the cytoplasm. These two facts led us to propose that

APS reduction by menaquinol has to be driven by another more favorable reaction, possibly involving flavin-based electron confurcation or bifurcation at QmoA or QmoB. Several examples of flavin based electron bifurcation have emerged in the last years, clearly demonstrating the importance of this mechanism in the energy metabolism of anaerobes and their bioenergetic evolution (Herrmann *et al.* 2008; Li *et al.* 2008; Schut and Adams 2009; Costa *et al.* 2010; Wang *et al.* 2010; Bertsch *et al.* 2013). The common features are the flavin cofactor, as the two electron carrier responsible for the bifurcation, and the involvement of the low redox potential Fd.

The direct electron transfer between Qmo and APS reductase was initially tested by spectrophotometric assays and by sulfite quantification methods. In all cases, direct electron transfer or sulfite formation was not observed. The reverse direct electron transfer, based on the reverse activity of AprBA, was also not observed. Assays with membrane extracts were also tested but the possibility of sulfite consumption by DsrAB made us exclude this method. We then explored confurcation reactions with *D. desulfuricans* cell extracts and with the pure proteins following menadiol oxidation and NADH oxidoreductase activity. Again no electron transfer was evident. With sulfite detection by the HPLC method we could, for the first time, see evidence of electron transfer through sulfite production over time (Figure 3.6, Reaction 1). However, sulfite production was observed when just the soluble proteins were present, as well as when Fd was absent, and thus did not support the proposal of confurcation. These results showed that the POR system is sufficient to

transfer electrons for APS reduction, but *in vivo* we know that QmoABC is required.

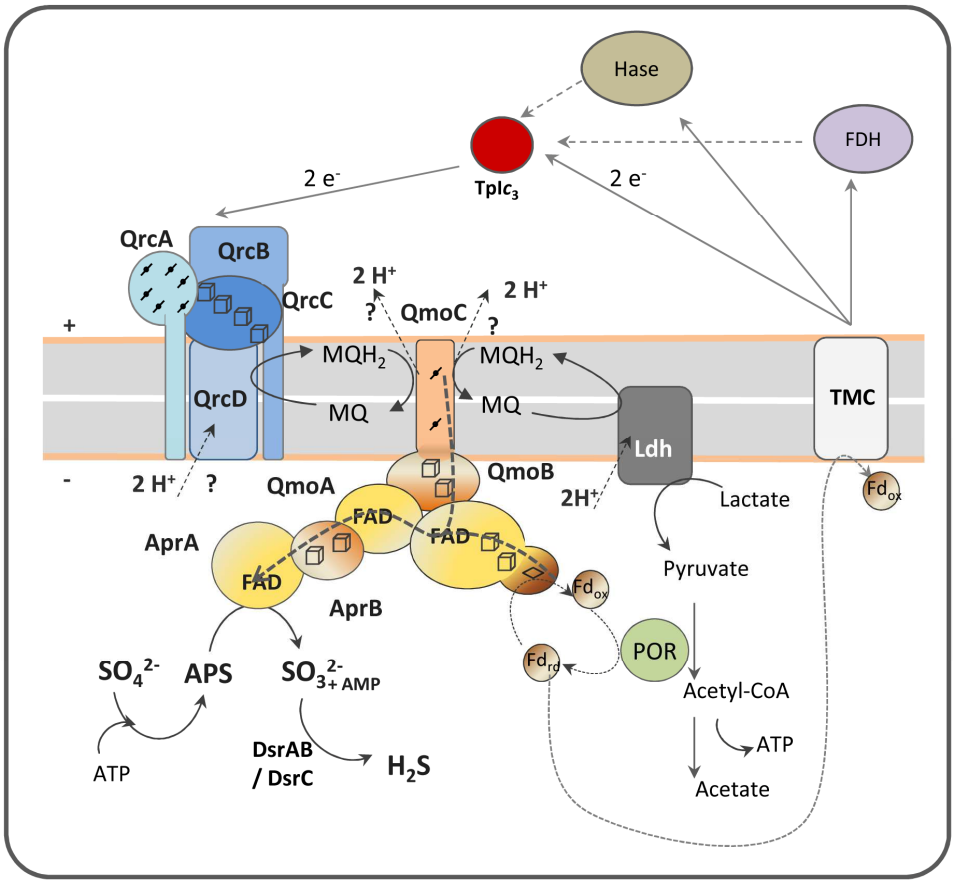
POR is responsible for the irreversible decarboxilation of pyruvate in the presence of coenzyme A in anaerobic organisms. The reaction generates low redox potential electrons that are used to reduce Fd or alternatively flavodoxin (Pieulle *et al.* 1995; Charon *et al.* 1999). POR from *D. africanus* is a homodimer, and each subunit contains a thiamine pyrophosphate (TPP) cofactor, two Fd type [4Fe-4S]$^{2+/1+}$ clusters and one [4Fe-4S]$^{2+/1+}$ cluster coordinated by an unusual cysteine sequence motif (clusters midpoint potentials: –390 mV, –515 mV and –540 mV) (Charon *et al.* 1999). POR interacts with Fd forming a complex that makes electron transfer possible, and is supported by kinetic and structural studies. Most PORs electron transfer happens to topologically equivalent species, such as Fds and Fd-like domains (Charon *et al.* 1999). The structure of the β-subunit of APS reductase (AprB) is divided in three segments and the folding of one of them is highly similar to that found in bacterial Fds and holds two [4Fe-4S]$^{2+/1+}$ clusters (Sticht and Rosch 1998). One of the AprB clusters is deeply buried in the protein matrix (Cluster I, $E^{0'}$ = –57 mV), while the other cluster is located at the protein surface and is more exposed to the solvent (Cluster II, $E^{0'}$ = – 520 mV) (Parey *et al.* 2013). The redox potential of cluster II is close to the redox potentials of Fds (~–400 mV), and additionally the cluster is more exposed which may explain the possibility of direct electron transfer from POR to APS reductase. Similarly, the bifurcating [FeFe] hydrogenase from *Thermotoga maritima* was able to receive electrons directly from POR, probably through the Fd-like delta subunit of POR (Schut and Adams 2009). These

considerations may explain the results obtained for Reaction 3 and we must also consider that these are *in vitro* experiments.

We also explored the possibility of electron bifurcation. The first bifurcation hypothesis considered that QmoB reduced by menaquinol could bifurcate electrons to QmoA/AprBA and to a second electron acceptor with a high redox potential. The problem of this proposal is that in SRB there is no candidate with high enough redox potential to drive this reaction, as it was discussed in the previous section. In the second bifurcation hypothesis we tested if Fd-reduced QmoB could bifurcate electrons to menaquinone and to QmoA/AprBA to reduce APS (Table 3.6, reaction 4). In this hypothesis reduced Fd (– 400 mV) is the electron donor and APS (– 60 mV) and menaquinone ($E^{0'}$ = – 75 mV), the electron acceptors. However, no sulfite production was detected in this reaction. Moreover, the control without menadione was able to produce traces of sulfite, so menadione possibly inhibits the reaction. Recent results in a *D. alaskensis* G20 strain lacking TpIc_3 cytochrome (Keller *et al.* 2014) provides evidence for the need of reduced menaquinol pool for APS reduction. In Keller and co-workers work, the growth of c_3 mutant on pyruvate-sulfate was impaired in contrast to lactate-sulfate growth. Growth of the mutant during lactate oxidation is not affected because lactate dehydrogenase reduces the menaquinone pool, while in pyruvate that is not possible in the absence of TpIc_3 cytochrome. Keller *et al.* concluded that electrons from pyruvate oxidation travel from the cytoplasm to the periplasm via a transmembrane complex, which in turn reduces TpIc_3 (Scheme 3.2). When sulfate is available as terminal electron acceptor, reduced TpIc_3 transfers electrons to the Quinone

reductase complex (QrcABCD) reducing the menaquinone pool which then reduces Qmo. The QrcABCD complex was isolated and characterized from *D. vulgaris* and is composed of a hexaheme cytochrome *c* subunit, a large subunit of the molybdopterin-containing protein family, a periplasmic iron-sulfur protein and an integral membrane protein of the NrfD family (Venceslau *et al.* 2010). The complex is involved in electron transfer from periplasmic hydrogenases and formate dehydrogenases through TpIc_3, and it has been proposed that together with Qmo, is responsible for the formation of a redox loop that contributes to the proton motive force (pmf) during sulfate reduction with H_2 or formate (Venceslau *et al.* 2010). (Keller *et al.* 2014) also proposed reduced Fd, generated from POR, as the low electron donor to Qmo in an electron confurcation mechanism for APS reduction, thus supporting our proposal (Scheme 3.2).

Finally, a possible problem with the described experiments is the absence of a membrane potential that is an essential component in respiratory electron transfer chains. In any biological cell the membrane allows the generation of ions gradients (ΔpH in case of protons and ΔNa for sodium ions) and also charge separations (Δψ, electrical potencial), that together contribute to the pmf (Simon *et al.* 2008). The QmoABC is a membrane bound protein, with a unique assembly of modules in the membrane QmoC subunit: one of its domains is a transmembrane cytochrome *b* and the second is a soluble electron transfer domain with two [4Fe-4S] clusters. The hemes are reduced by menaquinol analogs (Pires *et al.* 2003), and QmoC must be involved in the electron transfer

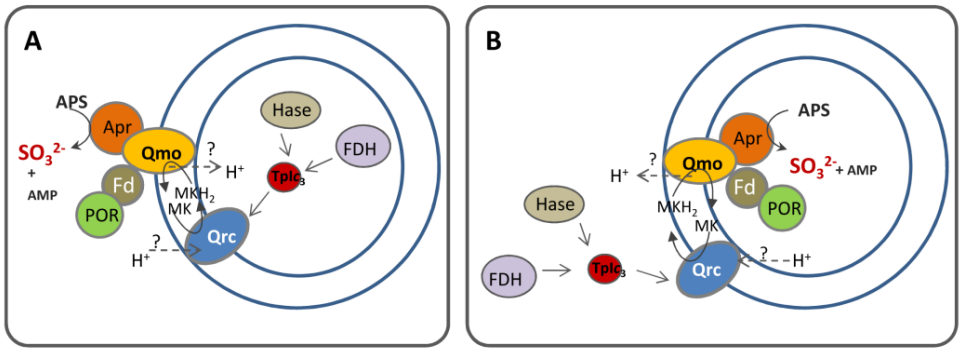


Scheme 3. 2 - Proposed electron confurcation mechanism for APS reduction, when lactate or pyruvate are electron donors for sulfate reduction, adapted from (Ramos *et al.* 2012) and (Keller *et al.* 2014). Menaquinol is produced either by lactate dehydrogenase during lactate oxidation or by electrons that are transferred from Tp*Ic*$_3$ to QrcABCD. TMC, generic transmembrane complex; MQ, menaquinone.

from the quinone pool to the cytoplasm, against the membrane potential. The *in vitro* experiments have always detergent present in order to maintain the integrity of the membrane complex, but this can constitute a disadvantage when studying possible electron transfer from

the quinone pool to the cytoplasm, as the detergent can hinder possible physical interactions between proteins, and proteins and quinone analogs, although that was not a problem in the previous section when protein-protein interaction studies were performed. In addition, quinols are small liposoluble organic molecules (Simon *et al.* 2008), emphasizing once more the importance of the membrane environment. So, the lack of an appropriate membrane environment can influence the results, preventing the electron transfer activity.

The reconstitution of membrane proteins in vesicles is a successful approach to study electron transport enzymes, as for example the case of the methanogenic respiratory chain in *Methanosarcina mazei* (Welte *et al.* 2010; Welte and Deppenmeier 2011b), fumarate respiration in *Wolinella succinogenes* (Biel *et al.* 2002), or to study the mechanism of action of membrane proteins (Unden and Kroger 1986; Rigaud and Levy 2003; Welte and Deppenmeier 2011a). Thus, in the present case it may be important to reconstitute this system in vesicles in order to create a more physiological environment. The membrane reconstitution system could include a menaquinone recycling system using, for example, the Qrc complex (Scheme 3.3). The reconstitution system should include the membrane proteins in two possible orientations with TpIc_3 and a Hase/FDH as periplasmic electron donors, and the cytoplasmic environment should be recreated with AprBA, Fd, POR (reduced with pyruvate and CoA) and APS (Scheme 3.3).



Scheme 3.3 - Schematic representation of the membrane reconstitution system in vesicles with the menaquinone recycling system with Qmo and Qrc. (A) Inside out vesicles reconstitution; (B) Right side out reconstitution of vesicles.

However, although the Qrc complex is well characterized and has TpIc_3:menaquinone oxidoreductase activity, the Qmo does not have a measurable enzymatic activity. We have tested three possible electron acceptors for menaquinol oxidation activity with no success due to direct reduction of these acceptors by the quinols. The absence of a reliable enzymatic assay for Qmo is a key problem in reconstituting the system in liposomes, because we need to know the amount of complex reconstituted and its orientation (Rigaud and Levy 2003). Clearly, more electron acceptors have to be tested.

In conclusion, different electron transfer experiments were used to test electron transfer from menaquinol to APS reduction through QmoABC-AprBA, either directly or by flavin-based electron bifurcation/confurcation mechanisms. The best method involved sulfite quantification by HPLC. We could detect sulfite formation in the confurcation assay involving Qmo, Fd reduced by POR system and

menadiol, but a similar electron transfer was observed directly from the POR system to AprBA, possibly from interaction between Fd-like domain in POR and AprB subunit. The reconstitution of Qmo in liposomes may be a good strategy for future experiments to understand how electrons flow from the membrane to the cytoplasm in the dissimilatory sulfate reduction (Scheme 3.3). However, it would be important to first develop a method to quantify the menaquinol oxidation activity of Qmo.

3.2.6 - AKNOWLEDGEMENTS

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CHAPTER 4

THE *HDRABC-FLOXABCD* GENE CLUSTER ENCODES A NOVEL NADH DEHYDROGENASE/HETERODISULFIDE REDUCTASE WIDESPREAD IN ANAEROBIC BACTERIA AND INVOLVED IN ETHANOL METABOLISM IN *DESULFOVIBRIO VULGARIS* HILDENBOROUGH

This Chapter will be submitted as:

Ramos AR, Grein F, Oliveira G, Venceslau SS, Keller KL, Wall JD and Pereira IAC "The *hdrABC-floxABCD* gene cluster encodes a novel NADH dehydrogenase/heterodisulfide reductase widespread in anaerobic bacteria and involved in ethanol metabolism in *Desulfovibrio vulgaris* Hildenborough"

4.1 - SUMMARY

The Flavin-based electron bifurcation mechanism (FBEB) is a recently recognized mechanism that is important for the energy metabolism of anaerobic Bacteria and Archaea. Here, we report on a new family of proteins, the **Fl**avin **ox**idoreductase (FloxABCD), which is a new NADH dehydrogenase that, together with a heterodisulfide reductase (HdrABC), seems to be involved in FBEB in the energy metabolism of the sulfate reducing organisms (Pereira *et al.* 2011). The *hdr-flox* gene cluster is widespread among many Bacteria with representatives *in Chlorobi, Proteobacteria, Firmicutes, Bacteroidetes, Spirochaetes, Fusobacteria*, *Actinobacteria* and *Acidobacteria* phyla, pointing for a general and important role in the energy metabolism of anaerobes. In this work we studied the FloxABCD proteins of the sulfate reducing organism *Desulfovibrio vulgaris* Hildenborough. Expression studies reveal that the *hdr-flox* genes are more expressed when *D. vulgaris* wild type cells are grown in ethanol-sulfate, followed by fermentative conditions with pyruvate. Two mutant strains were generated, one containing a Ω kanamycin (Km) cassette insertion in *hdrC*, and another lacking the *floxA* gene. Both strains were unable to grow in ethanol-sulfate medium, and growth was restored in a *floxA*-complemented strain. In addition, these strains produced reduced amounts of ethanol from pyruvate fermentation, compared to the wild type, revealing their role in reducing NAD^+ for ethanol production, while recycling Fd_{red}. Our results show that in *D. vulgaris* the FloxABCD-HdrABC proteins are essential for growth on ethanol, probably involving a FBEB mechanism that leads to Fd and DsrC

reduction, while in fermentation they operate in reverse, reducing NAD^+ for ethanol production.

4.2 - INTRODUCTION

The increasing number of sequenced genomes together with high throughput approaches and improved biochemical and genetic studies, are providing considerable progress in our understanding of the bioenergetic metabolism of prokaryotic anaerobes revealing an unexpected diversity (Deppenmeier and Müller 2008; Muyzer and Stams 2008; Thauer *et al.* 2008; Biegel *et al.* 2011; Mahadevan *et al.* 2011; Pereira *et al.* 2011; Buckel and Thauer 2013; Schoepp-Cothenet *et al.* 2013). In the past few years a new mechanism for energetic coupling has been found to operate in anaerobes, the flavin-based electron bifurcation (FBEB), which is a process that allows the coupling of energetically unfavourable reactions, such as the reduction of ferredoxin, to energetically favourable ones (Herrmann *et al.* 2008; Thauer *et al.* 2008; Kaster *et al.* 2011; Buckel and Thauer 2013). This process is believed to have been present in the early forms of life on Earth (Buckel and Thauer 2013; Sousa *et al.* 2013). The mechanism of FBEB is analogous to the concept of quinone electron bifurcation that was introduced in 1976 by Peter Mitchell (Mitchell 1976). The FBEB reaction involves a flavin cofactor and the generation of a flavin-semiquinone intermediate. So far, FBEB has been demonstrated experimentally in seven biological reactions present in fermenting clostridia (Herrmann *et al.* 2008; Li *et al.* 2008; Wang *et al.* 2010; Wang *et al.* 2013a; Wang *et al.* 2013b), in fermentative hyperthermophilic

bacteria (Schut and Adams 2009), in acetogenic bacteria (Schuchmann and Müller 2012; Bertsch *et al.* 2013) and in methanogens (Thauer *et al.* 2008; Costa *et al.* 2010; Kaster *et al.* 2011). In all cases, the low potential electron donor ferredoxin plays a central role in the process, working as energy and redox currency in the cell, and its oxidation is linked to chemiosmotic energy conservation or is used to drive key catabolic processes (Herrmann *et al.* 2008; Buckel and Thauer 2013). A prototypical example of FBEB occurs in hydrogenotrophic methanogens, where a complex between the HdrABC heterodisulfide reductase and the MvhADG hydrogenase is responsible for regenerating the two reduced thiol cofactors CoM and CoB in the last step of methanogenesis (Thauer *et al.* 2008). The bifurcation reaction couples the reduction of ferredoxin with H_2 (endergonic reaction) to reduction of the CoM-S-S-CoB heterodisulfide with H_2 (exergonic reaction) (Thauer *et al.* 2008; Kaster *et al.* 2011; Buckel and Thauer 2013). HdrA is the flavin containing subunit that is thought to be involved in the bifurcation of electrons (Thauer *et al.* 2008; Buckel and Thauer 2013).

Several proteins involved in FBEB seem to be widespread in prokaryotes, suggesting that electron bifurcation is a general mechanism for conserving energy in chemotrophic anaerobic bacteria (Martin 2011; Nitschke and Russell 2011; Buckel and Thauer 2013). In our genome analysis of 25 genome-sequenced sulfate reducing organisms (SRO; both Bacteria and Archaea) we found that there are several examples of soluble proteins in these organisms that may carry out FBEB, from H_2, NADH, formate or other carbon-based electron donors (Pereira *et al.* 2011). In particular, a group of proteins related to HdrA of methanogens

can be found in sulfate reducers, suggesting that electron bifurcation can be involved in their energy metabolism. A set of *hdrABC* genes or an isolated *hdrA* gene are often found next to a set of *mvhADG* genes or to a set of newly recognized genes that were named *floxABCD* (for *fl*avin *ox*idoreductase), in many of these organisms (Pereira *et al.* 2011). Moreover, there are proteins directly involved in sulfate reduction that share similarity to HdrA, namely the QmoA and QmoB flavin subunits of the QmoABC membrane complex that is essential for sulfate reduction (Zane *et al.* 2010), and is believed to be the electron donor to AprBA (Pires *et al.* 2003; Ramos *et al.* 2012). Recently, QmoABC was also proposed to perform flavin based electron confurcation (Ramos *et al.* 2012). This reverse bifurcation mechanism couples the endergonic reduction of APS by menaquinol to the exergonic reduction of APS by a low-potential soluble electron donor (most likely ferredoxin) (Ramos *et al.* 2012).

In this work we focus on the FloxABCD proteins that correspond to a new putative NADH dehydrogenase complex identified in SRO, encoded next to genes coding for HdrABC (Pereira *et al.* 2011). The FloxABCD-HdrABC complex is analogous to the MvhDGA-HdrABC complex of methanogens where the Mvh and Flox proteins probably constitute parallel pathways for HdrA reduction from H_2 or NAD(P)H, respectively. The important function of FloxABCD-HdrABC in the energy metabolism of the deltaproteobacterium *Desulfovibrio vulgaris* Hildenborough is revealed by the fact that their genes are often implicated in gene expression and proteomic studies of *D. vulgaris* energy metabolism (Haveman *et al.* 2003; Zhang *et al.* 2006b; Caffrey *et al.* 2007; Pereira *et al.* 2008; Walker

et al. 2009; Meyer *et al.* 2013; Meyer *et al.* 2014). Recently, the *hdr-flox1* genes of *Desulfovibrio alaskensis* G20 were proposed to be involved in oxidizing NADH in a new pathway leading to fumarate reduction (Meyer *et al.* 2014).

Here, we report on studies for a detailed characterization of the *hdr-flox* gene cluster present in *D. vulgaris*. Our results show that the FloxABCD-HdrABC complex is essential for ethanol oxidation in *D. vulgaris* Hildenborough, probably involving a FBEB mechanism. We also find evidence that in pyruvate fermentation these proteins are responsible for reducing NAD^+, leading to the production of ethanol as a fermentative product, while allowing for ferredoxin oxidation. These results provide the first detailed characterization of the function of the *hdr-flox* genes, which encode a new family of previously unrecognized NADH dehydrogenases widespread in anaerobic bacteria.

4.3 - Materials and Methods

4.3.1 - Genome and sequence analysis

Genomes were analyzed at the Integrated Microbial Genomes website. Multiple sequence alignments were performed with ClustalX2. Domain prediction was performed with InterPro and Pfam at the European Bioinformatics Institute web server. Secondary structure prediction was performed with several platforms including JPred, PredictProtein, SOSUI and SABLE. Putative transmembrane β-barrel domains were analyzed with PredTMBB.

4.3.2 - STRAINS AND MEDIA

The strains used in this work are listed in Table 4.1. *Escherichia coli* α-select stain was cultured in LB medium (per liter of medium: 10 g tryptone, 10 g sodium chloride and 5 g yeast extract). Where indicated, kanamycin or spectinomycin was added to LB medium to a final concentration of 50 µg/ml and 100 µg/ml, respectively.

All *D. vulgaris* strains were grown at 37 ^{o}C in MOY medium. MOY basal medium contained 8 mM magnesium chloride, 20 mM ammonium chloride, 0.6 mM calcium chloride, 2 mM potassium phosphate (dibasic), 60 µM ferrous chloride, 120 µM EDTA, 30 mM Tris-HCl (pH 7.4), 1 ml Thauer's vitamin solution, 1 g/liter of yeast extract and 6 ml trace element solution per liter. pH was adjusted to 7.2 with 1 M H_2SO_4. MOY medium was amended with sodium thioglycolate (1.2 mM final concentration) as reductant and resazurin (640 nM final concentration) as redox-potential indicator. Thauer's vitamin solution contains: 82 µM biotin, 45 µM folic acid, 486 µM pyridoxine hydrochloride, 148 µM thiamine hydrochloride, 133 µM riboflavin, 406 µM nicotinic acid, 210 µM *DL*-pantothenic acid, 365 µM *p*-aminobenzoic acid, 242 µM lipoic acid, 14 mM choline chloride and 7.4 µM vitamin B12. Trace element solution contains 2.5 mM manganese sulfate, 1.26 mM cobalt chloride, 1.5 mM zinc sulfate, 206 µM sodium molybdate, 323 µM boric acid, 378 µM nickel chloride, 11.7 µM cupric chloride, 23 µM sodium selenite and 27 µM sodium tungstate (Zane *et al.* 2010; Keller *et al.* 2011).

Sodium lactate (60 mM), sodium pyruvate (60 mM), ethanol (40mM), or hydrogen (1 bar) were added as electron donors and sodium sulfate (30mM for lactate and H_2 growth, 20 mM for ethanol) or sodium sulfite

(20 mM) were added as terminal electron acceptors. For growth in pyruvate a small amount of sulfate (2 mM) had to be present in the beginning, or growth was not observed. Replacing sulfate with sulfide was not effective. Hydrogen growth medium contained sodium acetate (10 mM), and was gassed with a mixture of 80% (v/v) H_2 and 20% CO_2; flasks were incubated in a horizontal position to increase the gas-liquid surface area.

Antibiotics were added to the MOY medium as follows: G418 at 400 µg/ml or spectinomycin at 100 µg/ml. G418 was used in place of kanamycin as described by Zane *et al*. 2010. For solidified MOY medium, 15 g agar per liter was added.

4.3.2.1 - PLASMIDS AND STRAINS CONSTRUCTION

The pMOIP11 plasmid for insertion of ΩKm cassette in *hdrC* was constructed by PCR, amplifying the region around *hdrC* from chromosomal DNA using primers #1 and #2 (Table 4.2) and the resulting fragment was ligated with the 2,564 bp *Xba*I/*Eco*RI fragment from pMO719 giving plasmid pMOIP10. The ΩKm cassette was cut from pHP45ΩKm using *Eco*RI and blunt ends were created using Klenow fragment (Fermentas). The resulting fragment was ligated into the *Pst*I site located in *hdrC* in pMOIP10 giving origin to pMOIP11. This plasmid was electroporated into *D. vulgaris* according to Keller *et al.* (2011), Zane *et al.* (2010), from which strain IPFG01 was obtained, by selecting with MOYLS4 medium containing G418 (*hdrC*::ΩKm). The genotype of IPFG01

Table 4.1 - List of strains and plasmids used in this work.

Strain or plasmid	Genotype or relevant characteristics	Source and/or reference
***E. soli* strains**		
α-Select (Silver efficiency)	*F⁻ deoR endA1 relA1 gyrA96 hsdR*17(r_k^- m_k^+) *supE*44 *thi*-1 Δ(*lacZYA-argFV*169) Φ*80δlacZ*ΔM15 *λ*⁻	Bioline
***D. vulgaris* strains**		
ATCC 29579	WT *D. vulgaris* Hildenborough	ATCC
IPFG01	WT Δ*hdrC*::ΩKm	This work
IPFG02	WT Δ*floxA*::Km^R	This work
IPFG03	WT Δ*floxA*::Km^R + pMOIP12P	This work
IPFG04	WT FloxA-Strep tag	This work
Plasmids		
pMO719	pCR8/GW/TOPO containing SRB replicon (pBG1); $Spec^R$	(Keller *et al.* 2009)
pSC27	*Desulfovibrio* shuttle vector; source of *aph(3')-II*; Km^R	(Rousset *et al.* 1998)
pHP45ΩKm	Source of ΩKm casette	(Fellay *et al.* 1987)
pPR-IBApelB	Strep-Tag C-terminal, Amp^R	(Grein *et al.* 2010)
IBAFloxAStrep	Expression of FloxA with C-terminal Strep Tag, Amp^R	This work
pMOIP05	*qmoA* expression vector with STF-Tag, $Spec^R$	(Ramos *et al.* 2012)
pMOIP10	pUC origin, region around *hdrC* $Spec^R$	This work
pMOIP11	pMOIP10 with ΩKm cassette in EcoRV site in HdrC, $Spec^R$, Km^R	This work
pMOIP12	pUC origin, FloxA1Strep, Kan^R $Spec^R$	This work
pMOIP12P	FloxA with C-terminal Strep Tag in pMOIP05 (replacing Insert) for **p**lasmid encoded tagging, $Spec^R$, Km^R	This work
pMOIP12C	FloxA with C-terminal Strep Tag in pMOIP12 (replacing Insert) for **c**hromosomal tagging, $Spec^R$, Km^R	This work
pMOIP14	intermediate plasmid to do pMOIP15/*floxA* downstream region in pMOIP13 (HindIII/XbaI), $Spec^R$	This work
pMOIP15a	*floxA* deletion plasmid/Kan gene in HindIII site, $Spec^R$, Km^R	This work

was verified by colony PCR using primers #1 and #2 and by Southern Blot. The pMOIP14 plasmid, an intermediate plasmid for pMOIP15a, was constructed by PCR amplifying the upstream and downstream regions of *floxA* (primers #3/ #4 and #5/ #6) and subsequently the two fragments were ligated in pMOIP11 replacing the original insert. The kanamycin resistance gene was amplified from pSC27 using primes #7 and #8 and inserted into pMOIP14 using *Hin*dIII. Since only one enzyme was used for the cloning, the *kan* gene can be inserted in two orientations. The plasmid chosen, pMOIP15a, has the *kan* gene in the same orientation as the up- and downstream genes of *floxA*. The plasmid was electroporated into *D. vulgaris* giving strain IPFG02 (Δ*floxA*::Km^R). The genotype of this strain was verified by colony PCR using primers #9 and #8 and by Southern blot.

The IBAFloxAStrep plasmid was created amplifying the *floxA* gene from chromosomal *D. vulgaris* Hildenborough DNA using primers #14 and #18 and cloning the PCR product into pPR-IBApelB (Grein *et al.* 2010), using the restriction endonucleases *Nde*I and *Eco*47III. To generate pMOIP12P, tagged *floxA* gene was amplified from IBAFloxAStrep using primers #14 and #15 and the product was inserted into pMOIP05 using *Nde*I and *Eco*RI replacing the original insert. This plasmid was electroporated in IPFG02 and selected with MOYLS4 medium containing G418 and spectinomycin. The complemented strain IPFG03 (Δ*floxA*::Km^R + pMOIP12P) was confirmed by PCR amplification of the insert and by sequencing performed in GATC Biotech, Germany. The pMOIP12C plasmid was created by cutting tagged *floxA* gene from IBAFloxAStrep using *Nde*I and *Hind*III and ligating it into a SLIC generated plasmid

containing 810 bp of the downstream region of *floxA*, the kanamycin resistance cassette from pSC27 and the pUC origin and spectinomycin gene from pMO719. The pMOIP12C plasmid was electroporated in *D. vulgaris* resulting in strain IPFG04, which was confirmed by colony PCR using primers #9 and #16.

Table 4.2 - Primers used for the plasmid and strains construction.

	Primer	Primer sequence (5' → 3')
#1	OmADHEco-f	TCT CCG AAT TCA ACC TCA TCG CCA AGC
#2	OmHdrBXba-r	TGA AGT CTA GAA CAC CGG CAT GTC CA
#3	Floxup-f	GCC TTT TGC TGG CCT TTT GCT CAC ATA TGT GAC CAC ACT CGG GTC
#4	Floxup-r	CCC ACT GCA AGC TAC CTC T AAG CTT ACT TTT CGA ACT GCG
#5	Floxdo-f	GCC TTC TTG ACG AGT TCT TCT GAC CGC GAT GCA GGG CTG TC
#6	Floxdo-r	CGA GGC ATT TCT GTC CTG GCT GGG GAC ACC TCC ACT GCC AAA TGG
#7	KanHind-f	GTC AAG CTT ATG GAC AGC AAG CGA AC
#8	KanHind-r	TAG AAG CTT GGT CGG TCA TTT CG
#9	FloxAcheck-f	GCT TCC ACA TCT TCG ACC GG
#10	Amp-f	ATG TGG CGC GGT ATT ATC CCG TAT
#11	FloxStrep-r	AGC GCT CAA CTC GTT GGG
#12	Amp-r	ATA CGG GAT AAT ACC GCG CCA CAT
#13	FloxStrep-f	AAC GAG TTG AGC GCT TGG AGC
#14	FloxANde-f2	CTA CAT ATG CCT GAC GCC ATC AC
#15	FloxA1StrepEco-r	TCA GAA TTC GCA GCC GGA TCA AG
#16	Kan-r	TCA GAA GAA CTC GTC AAG AAG GC
#17	*hdrC*downF	ATC TCG CCT ACT ATC CCG G
#18	FloxANde-r	AAT AGC GCT CAA CTC GTT GGG CAG

4.3.2.2 - SOUTHERN BLOT

Southern blot analysis of strains IPFG01, IPFG02 and IPFG03 was performed according to the instructions in (Keller *et al.* 2011). Genomic

DNA of the three strains were digested with *Bci*VI, and for IPFG01 strain a *hdrC* downstream probe was generated with primers #2 and #17 (Table 4.2), while for IPFG02 and IPFG03 strains a *floxA* upstream probe was created with primers #5 and #6. A DNA band of 4,182 bp showed hybridization in the wild type in contrast to a fragment of 2,890 bp in IPFG01, confirming the mutation. The two other strain, IPFG02 and IPFG03 presented a DNA fragment of 6,340 bp in contrast with the wild type fragment of 8,807 bp. Southern blotting, prehybridization and hybridization was performed with DIG High Prime labeling and Detection Starter kit II from Roche, according to the manufactures protocol. Results are in Supplementary material.

4.3.3 - GROWTH CURVES

D. vulgaris Hildenborough WT and mutant strains were grown anaerobically at 37 ^{o}C in 100 ml flasks with 50 ml of medium. All media were inoculated with 2% (v/v) fresh precultured cells grown in lactate-sulfate medium (LS4), except for the hydrogen-sulfate (HS4) growth where 10% (v/v) inoculum was used. The optical density (OD at 600 nm) of the cultures was monitored at various time points with a spectrophotometer Shimadzu UV-1603. All reported optical density measurements are the mean of four biologically independent experiments.

4.3.4 - ETHANOL QUANTIFICATION

Ethanol accumulation in the growth media was determined with an enzymatic kit from NZYTech. This method is based in the NADH formed

from ethanol through the combined action of Adh and aldehyde dehydrogenase.

4.3.5 - Western Blot analysis of FloxA, HdrA and Adh gene expression

D. vulgaris cells grown in lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), ethanol-sulfate (ES4) and hydrogen-sulfate (HS4), were collected at two different time points, mid-exponential and stationary phase, and centrifuged for 12 min at 3,000 *g*. Cells were then disrupted using the BugBuster® Protein Extraction Reagent (Novagen®) for 20 minutes at room temperature and centrifuged at 16,000 *g* for 20 minutes at 4 ^{o}C. The protein concentration in the cell extracts was determined by the Bradford method (BioRad) with bovine serum albumin as the standard (NZYTech).

Soluble crude extracts (25 µg) were analyzed in SDS-PAGE gels [12% acrylamide (v/v)] and transferred to polyvinylidene difluoride (PVDF) membranes (transfer buffer: 48 mM Tris-HCl pH 9.2 and 39 mM Glycine) using a Mini Trans-Blot ® electrophoretic transfer cell (BioRad) during 30 min at 4 ^{o}C, 100 V and 350 mA. The membranes were treated with blocking buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20 (v/v) and 5% non-fat milk (w/v)), overnight at room temperature. The next day, after two washing steps with TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl , 0.05% Tween 20 (v/v)) anti-FloxA antibody at 1:1 000 dilution in TBST, anti-HdrA antibody at 1 : 500 or anti-Adh at 1:5 000 dilution in TBST were incubated with the membranes for 1 h at room temperature; after two washing steps with TBST, membranes were incubated with anti-rabbit IgG antibody (Sigma-Aldrich®) at 1:15 000 dilution in TBST for

45 minutes. After three washing steps with TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl), protein detection was performed with Alkaline Phosphatase Buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) and NBT (nitro-blue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Carl Roth®). *D. vulgaris* antibodies against FloxA and HdrA subunits were produced in rabbits with artificial synthesized peptides (Table 4.3) and Adh antibody was produced with pure protein, at Davids Biotechnologie GmbH®.

Table 4.3 - Peptide sequence used to generate antibodies against FloxA and HdrA by artificial peptide synthesis.

Antibody	Peptide sequence
FloxA	DGPVFSYAELKELPNEL
HdrA	LQHFTDNQILAEVNALCLS

4.3.6 - QUANTITATIVE REAL-TIME PCR

FloxA, HdrA and Adh (DVU2405) gene expression was analyzed by quantitative real-time PCR (qRT-PCR) of *D. vulgaris* cells grown in lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), ethanol-sulfate (ES4) and hydrogen-sulfate (HS4). Cells from three independent experiments were collected at two different time points, mid-exponential and stationary phase, centrifuged for 12 min at 3,000 *g*, washed with cold (4 ^{o}C) sterile MilliQ water and frozen for later RNA extraction. Total RNA was extracted as previously described (Silva *et al.* 2001). DNase treatment was performed with Turbo Dnase (Ambion) in order to avoid genomic DNA contamination in the RNA extracts and was also followed by a RNA

clean-up kit (Qiagen). cDNA synthesis from each RNA sample (1µg) was performed using Transcriptor Reverse Transcriptase (Roche).

Primers were designed to generate amplicons of ~100 bp for FloxA, HdrA and Adh (DVU2405), and the 16S rRNA gene (Table 4.4). Reverse Transcriptase quantitative PCR reactions were performed in a Light Cycler 480 Real-Time PCR System (Roche), with Light Cycler 480 SYBR Green Master I (Roche). Relative standard curves and gene expression were calculated by the relative quantification method with efficiency correction, using the LightCycler Software 4.1, using 16S rRNA gene as a reference. For the final results three biological replicates and two technical replicates were used for each condition.

Table 4.4 - Primers used in qRT-PCR to determine the relative expression of *D. vulgaris* Adh (DVU2405), FloxA and HdrA.

Target	Primer sequence (5' → 3')
Adh (DVU2405)	Forward:ACCAAGAACGCGCAGAA
	Reverse: CGGTTCTGTCTGTACTCCTTAC
FloxA	Forward:ACCAAGTACGTGTGTGTCG
	Reverse:CTGCATCGCGGCTACAA
HdrA	Forward: CATTCCCAAGAAGGCGATCA
	Reverse: CGACAATCTCATCCTCCATGTC
16S RNA	Forward: CCTATTGCCAGTTGCTACC
	Reverse: AAGGGCCATGATGACTTGAC

4.3.7 - PROTEIN PURIFICATION

Cells of *D. vulgaris* WT were grown in lactate-sulfate containing medium, and IPFG04 cells were grown in MOY basal medium with lactate or ethanol as electron donors and sulfate as electron acceptor. The cells

were broken and centrifuged in anaerobic conditions and the soluble fraction was used to attempt purification of the FloxABCD-HdrABC complex. The purifications were conducted inside a Coy anaerobic chamber [95% (v/v) N_2, 5% (v/v) H_2] using an AKTA™ Prime plus™ system. The purification was monitored following the NADH oxidation activity with potassium ferricyanide and by Western blot with FloxA and HdrA antibodies.

WT soluble faction was purified in a Q-Sepharose HP column equilibrated with 50 mM Tris-HCl (pH 7.6) buffer containing 10% (v/v) glycerol and 5 µM FAD (Buffer A). A step gradient of increasing NaCl concentration was performed with Buffer A containing 1 M NaCl, and fractions were separated according to UV-visible spectra. The fractions with higher NADH activity and positive for FloxA were further purified on a second Q-Sepharose HP column followed by gel filtration in a Superdex® 200 (GE Healthcare) column equilibrated with Buffer A containing 150 mM NaCl and 5 µM FAD. *D. vulgaris* WT soluble fraction was also purified in more acidic conditions in 50 mM phosphate buffer (pH 6.5) containing 10% (v/v) glycerol, 5 µM FAD and 5 mM $MgCl_2$ (Buffer B). The soluble extract was first loaded in a Q-Sepharose HP column, and the fraction with more activity and positive for FloxA was applied in a HiTrap™ Phenyl HP (GE Healthcare) equilibrated in Buffer B with 1 M ammonium sulfate.

The IPFG04 strain encodes a Strep-tagged FloxA version in the chromosome. Three different purifications approaches were attempted with this strain. Cells grown either in LS4 or ES4, were disrupted, and the soluble crude extract was used for either affinity purification, anionic exchange chromatography (Q-Sepharose HP) followed by affinity

purification or anionic exchange chromatography (Q-Sepharose HP) followed by hydrophobic interaction chromatography (HiTrap™ Phenyl HP). The affinity purification was performed in gravity columns containing Strep®-Tactin resin (IBA GmbH) equilibrated with 50 mM Tris-HCl pH 7.6, 150 mM NaCl and 10% glycerol (v/v) (Buffer W). After loading the soluble crude extract, five washing steps with Buffer W were performed. The Strep-tagged protein was eluted with Buffer W containing 2.5 mM desthiobiotin. The anionic exchange and hydrophobic interaction chromatography were as described for *D. vulgaris* soluble fraction.

Adh was purified from the soluble fraction of IPFG04 cells grown in ethanol, using first a Q-Sepharose HP according to (Hensgens *et al.* 1993). NaCl was added to the fraction with Adh activity, to a concentration of 1 M. The fraction was then purified in a HiTrap™ Phenyl HP (2 ml column volume, CV) equilibrated with 50 mM Tris-HCl (pH 7.6) and 1 M NaCl. The column was eluted with a linear gradient of 1 to 0 M NaCl (20 CV). Fractions with highest Adh activity eluted between 1 and 0.7 M NaCl. The identity of the enzyme purified was confirmed as Adh (DVU2405) by Mass spectrometry.

4.3.8 - ENZYMATIC ASSAYS

The assays were performed inside the anaerobic chamber (95% N_2, 5% H_2 atmosphere) with a Shimadzu UV-1800 spectrophotometer in a stirred cuvette (Hellma). NADH dehydrogenase activity was determined at 420 nm monitoring potassium ferricyanide reduction ($\varepsilon_{\text{potassium ferricyanide}}$ at 420 nm = 1.05 mM^{-1} cm^{-1}) in 20 mM Tris-HCl (pH 7.6), 200 µM NADH

and 1 mM $K_3Fe(CN)_6$, at room temperature (Gomes *et al.* 2001). Alcohol dehydrogenase activity was determined as NADH formation at 340 nm (ε_{NADH} = 6.22 mM^{-1} cm^{-1}) in 50 mM Tris-HCl (pH 9), 5 mM NAD^+ and 20 mM ethanol, at room temperature (Hensgens *et al.* 1993).

4.4 - RESULTS

4.4.1 - THE FLOXABCD PROTEINS

Our comparative genome analysis of energy metabolism genes present in SRO revealed that a set of genes that were named *floxABCD* (for *fl*avin *ox*idoreductase) were present in many of these organisms, as part of a gene cluster containing also *hdrABC* or in some cases only an *hdrA* gene (Pereira *et al.* 2011). In *D. vulgaris* Hildenborough the *floxABCD* genes (locus tags DVU2399-DVU2404) were previously identified as encoding a hydrogenase-heterodisulfide oxidoreductase (Hase:Hdr) (Haveman *et al.* 2003), as the *flox* genes are homologous to subunits of *Pyrococcus* soluble hydrogenases (Jenney and Adams 2008). However, since no gene coding for a catalytic hydrogenase subunit is present, FloxABCD does not encode a hydrogenase (Pereira *et al.* 2011).

In *D. vulgaris* the *floxABCD* genes (locus tag DVU2399-DVU2401) code for only three cytoplasmic soluble proteins with 31 kDa (DVU2399; FloxA), 39 kDa (DVU2400; FloxB) and 54 kDa (DVU2401; FloxCD), as the *floxCD* genes are fused. The *floxA* gene codes for a protein with a FAD binding domain near the N-terminus (aa 19-119), a NAD(P)-binding domain (aa 123-226) and a domain binding one [2Fe-2S]-center (aa 248-276) (Figure

4.1). FloxA is homologous to soluble FAD/NAD(P)-binding oxidoreductases, including the γ subunit of *P. furiosus* soluble hydrogenases (SH) I and II (locus tags PF0892 and PF1330) that is responsible for NADPH oxidation. The *D. vulgaris* FloxA shares 39% identity and 51% similarity with *P. furiosus* SH I and 32% identity and 48% similarity with *P. furiosus* SH II – Figure 4.1, and Supplementary material. The *floxB* gene codes for an iron-sulfur electron transfer protein with a binding site for two canonical [4Fe-4S] centers (aa 250-344) near the C-terminus, and which contains four additional conserved cysteines that may bind another iron-sulfur center. The protein is homologous to the β subunit of *P. furiosus* SH I and II (PF0891 and PF1329) – 25% identity, 34% similarity with SH I and 26% identity and 39% similarity with SH II (Figure 4.1 and Supplementary material). In *D. vulgaris* and some other organisms, the *floxCD* genes are fused in a single gene coding for only one protein, with FloxC (C-terminus) and FloxD (N-terminus) domains. When not fused, *floxC* codes for a protein similar to FloxB and to the β subunit of *P. furiosus* SH I and II, with a domain binding two [4Fe-4S] centers, while *floxD* codes for a protein similar to the MvhD subunit of the MvhAGD hydrogenase of methanogens that contains a [2Fe-2S] cluster, and is involved in the interaction with HdrA (Stojanowic *et al.* 2003). The N-terminus domain of *D. vulgaris* FloxCD shares 44% identity and 58% similarity with MvhD from *Methanothermobacter marburgensis*. The C-terminus domain of *D. vulgaris* FloxCD shares 15% identity and 24% similarity with FloxB and is also homologous to the β subunit of *P. furiosus* SH I and II (PF0891 and

PF1329) – 12% identity, 21% similarity with SH I and 22% identity and 30% similarity with SH II (Figure 4.1 and Supplementary material).

So, comparing the general subunit composition of Flox with that of *P. furiosus* soluble hydrogenases and MvhADG hydrogenases, we can conclude that Flox will use NAD(P)H as electron donor/acceptor, that it includes two subunits (FloxB and FloxC) responsible for electron transfer, but that it does not have a hydrogenase catalytic subunit. Instead of this catalytic subunit Flox has an iron-sulfur subunit (FloxD), similar to MvhD, which is involved in electron transfer and interaction with HdrA. In fact, there are also many examples of HdrA-MvhD fusion proteins (Grein *et al.* 2013). So the FloxABCD proteins will transfer electrons between NAD(P)H/NAD(P)$^+$ and HdrABC, encoded in the neighbouring genes.

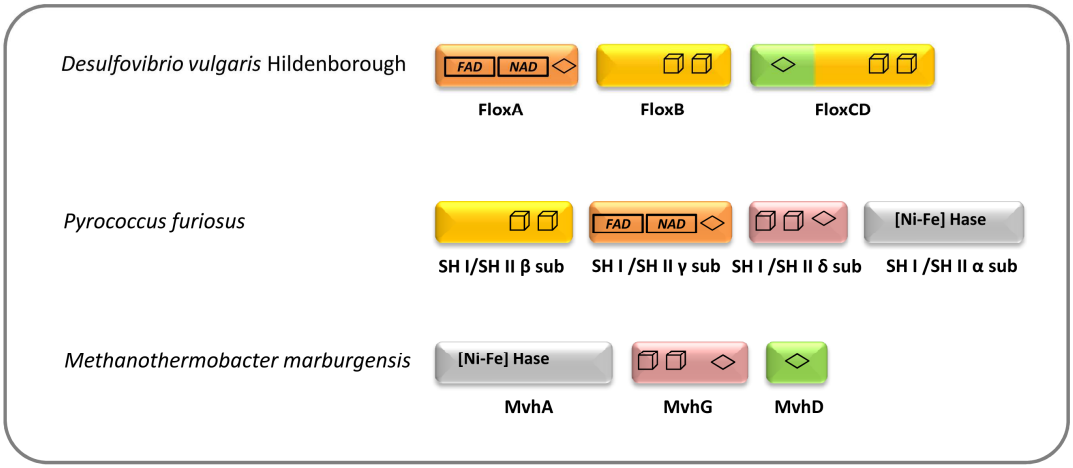

Figure 4.1 - Schematic representation of *D. vulgaris* Hildenborough Flox proteins, and related proteins from *Pyrococcus furiosus* and *Methanothermobacter marburgensis*. Identical colors denote similarity. Cubes - [4Fe-4S] clusters; diamond – [2Fe-2S] cluster.

4.4.2 - THE *HDRCBA-FLOXDCBA* GENE CLUSTER IS WIDESPREAD IN BACTERIA

We found that the *hdrCBA-floxDCBA* gene cluster is present in a large number of Bacteria (over 140), including members of many different phyla such as *Chlorobi, Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Spirochaetes, Actinobacteria* and *Acidobacteria* (Figure 4.2). We selected 17 organisms belonging to these different phyla for further analyses, including 5 *Deltaproteobacteria*, 5 *Firmicutes/Clostridia*, 2 *Chlorobi*, and one representative of *Bacteroidetes* (*Bacteroides cellulosilyticus* DSM 14838), *Fusobacteria* (*Fusobacterium varium* ATCC 27725), *Spirochaetes* (*Treponema brennaborense*), *Actinobacteria* (*Slakia heliotrinireducens*) and *Acidobacteria* (*Holophaga foetida* DSM6591) (Figure 4.2). The loci for the genes analyzed can be found in Supplementary material. The *hdrABC-floxABCD* gene cluster organization is strictly conserved among these different organisms. Interestingly, the *floxABCD* genes were not found in any archaeal organism, including methanogens. The FloxABCD proteins from the selected organisms were compared with the *D. vulgaris* proteins through sequence alignments (Supplementary material), revealing a strict conservation of the cofactor-binding sites. The *floxCD* genes are fused in *D. vulgaris*, whereas the *hdrCB* genes are fused in *Chlorobaculum tepidum* (former *Chlorobium tepidum*) and *Pelodictyum luteolum.* In some SRO only an HdrA (or closely related HdrL protein that includes also FAD and NAD(H) binding sites (Strittmatter *et al.* 2009; Grein *et al.* 2013) is found next to the *floxABCD* genes (Pereira *et al.* 2011). An example is *Desulfobacterium autotrophicum* (Figure 4.2). In *D. vulgaris* Hildenborough the *hdrABC-*

floxABCD genes are flanked by two alcohol dehydrogenase genes, one of which, DVU2405 *adh1*, is one of the most highly expressed genes in WT cells grown in lactate, pyruvate, formate or hydrogen as electron donors for sulfate reduction (Haveman *et al.* 2003). The *adh1* gene is probably not part of the same transcriptional unit as the *hdrABC-floxABCD* genes, as a putative hairpin loop was reported downstream of *adh1* (Haveman *et al.* 2003). In *Carboxydothermus hydrogenoformans* we can also find upstream to the *hdr-flox* genes an *adh* gene (Figure 4.2). Such a neighbouring *adh* gene is found close to the *hdr-flox* gene cluster in many *Desulfovibrio* spp., but it is absent in many other SRO and in the other anaerobes (see Figure 4.2 and Supplementary material).

Figure 4.2 - Genomic organization of the *flox* and *hdr* genes in selected Bacteria: *Deltaproteobacteria, Firmicutes/Clostridia, Chlorobi, Bacteroidetes, Fusobacteria, Spirochaetes, Actinobacteria* and *Acidobacteria*. ▼ - predicted promoter region; **Ω** – predicted terminator region.

4.4.3 - CO-EXPRESSION OF *FLOX* AND *HDR* GENES

Using online tools dealing with operon prediction (Chuang *et al.* 2012), namely the MicrobesOnline website (www.microbesonline.org) and Database of prOkaryotic OpeRons (DOOR), in *D. vulgaris* the *hdr-flox* genes are predicted to form an operon. Possible promoter and terminator regions where analyzed with Neural Network Promotor Prediction and TransTermHP (Kingsford *et al.* 2007), and upstream of the *adh-hdr-flox* genes there are three putative promoter regions, whereas downstream of these genes there is a putative terminator region (Figure 4.2). Thus, the *flox* genes are predicted to be in the same operon as the *hdr* genes. These predictions did not identify the reported hairpin loop downstream of *adh1* (Haveman *et al.* 2003), but expression studies (see below) confirm that expression of *adh1* is much higher than that of the *hdr-flox* genes.

To confirm that the *flox* and *hdr* genes are located in the same transcriptional unit, a strain was constructed containing a ΩKm cassette from plasmid pHP45ΩKm (Fellay *et al.* 1987) inserted into *hdrC*, the first gene of the cluster [strain IPFG01(*hdrC*::ΩKm)]. The ΩKm cassette leads to a premature termination of the transcription of *hdrC* and of the downstream genes within the same transcriptional unit. The genotype of strain IPFG01 was verified by colony PCR and Southern Blot (Supplementary material). We next checked by Western blot if FloxA was still expressed in IPFG01. *D. vulgaris* WT and IPFG01 cells were grown in MOYLS4 medium and the soluble crude extract of both strains was analyzed in SDS-PAGE gels followed by Western blot, using a FloxA antibody. FloxA was detected in the WT but not in the IPFG01 crude

extract (Figure 4.3), proving evidence that the *flox* genes are part of the same transcriptional unit as *hdrC*.

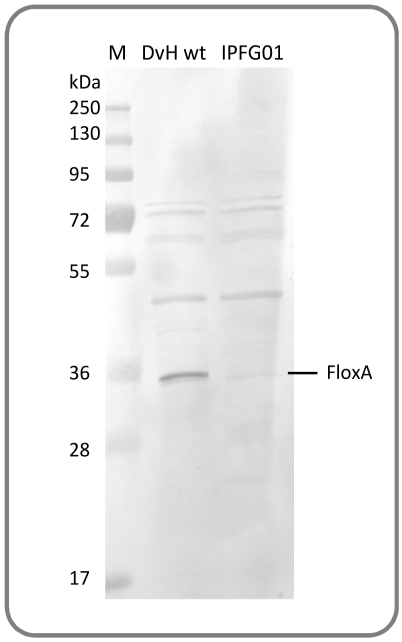

Figure 4.3 - Western blot of *D. vulgaris* and IPFG01 crude cell extracts using polyclonal antibodies against the FloxA subunit. Cells were grown with lactate-sulfate (LS4) for 18h, harvested and 25 µg of soluble crude extract was used for immunodetection.

4.4.4 - EXPRESSION STUDIES IN DIFFERENT GROWTH CONDITION

The relative gene expression of *floxA*, *hdrA* and *adh1* genes was measured by qRT-PCR at mid-exponential growth phase from cells grown in MOY media containing either lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), ethanol-sulfate (ES4) or hydrogen-sulfate (HS4) (Figure 4.4). The *hdrA* gene shows highest expression in ES4 followed by P conditions, and lowest expression in LS3 and HS4 conditions. A similar expression

behavior is observed for the *floxA* gene, except for the P condition, where the expression is lower than in LS4. The *adh1* gene also shows highest relative expression in ES4 and P conditions.

To complement these results, we analyzsed also the expression of FloxA, HdrA and Adh1 proteins by Western blot in *D. vulgaris* WT cells grown in the same conditions and collected at two time points: mid-exponential and beginning of the stationary phase (Figure 4.5).

The Western blot analysis confirms that the relative protein expression levels of FloxA, HdrA and Adh at middle exponential phase are highest in ES4 conditions. Again, HdrA and Adh1 also show strong expression in P conditions. Overall, there is good agreement between the mRNA levels and the protein levels for the three cases. However, Western blot cannot provide the relative expression levels of one protein to another. In the stationary phase the levels of FloxA decreases, except in ES4 conditions, whereas HdrA shows a strong decrease in all conditions. In contrast, Adh1 expression is even higher at the stationary phase for ES4 and LS4 conditions.

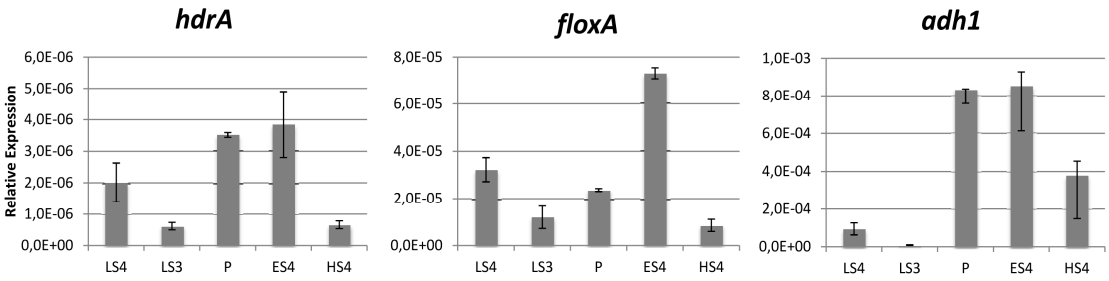

Figure 4.4 - Quantification of the mRNA levels of *hdrA, floxA* and *adh1* determined by qRT-PCR in mid-exponential phase *D. vulgaris* cells grown in lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), ethanol-sulfate (ES4) and hydrogen-sulfate (HS4). The relative expression levels are represented on the y axis, using the 16S rRNA gene as reference. The results shown are from three independent biological experiments, with standard errors.

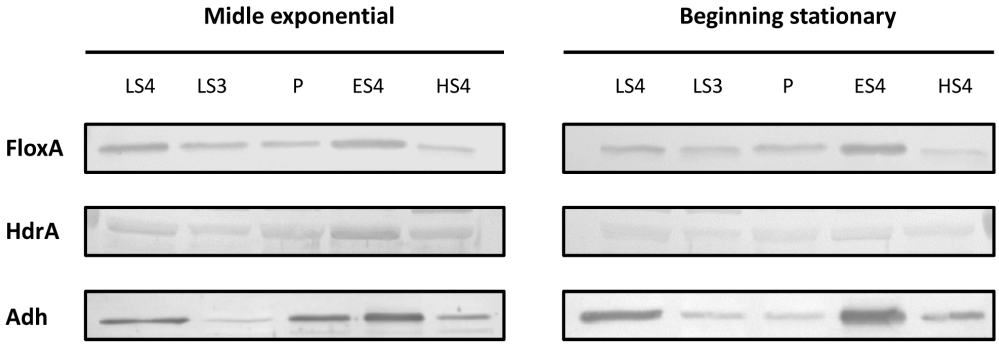

Figure 4. 5 - Western blots of *D. vulgaris* crude cell extracts with antibodies against FloxA, HdrA and Adh1. Cells were grown with lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), ethanol-sulfate (ES4) and hydrogen-sulfate (HS4), and harvested at two diferent time points, mid-exponential and beginning stationary phase. Crude soluble extract (25 µg) was used for immunodetection in all cases.

4.4.5 – GROWTH STUDIES OF MUTANT STRAINS

To evaluate the physiological function of the *flox* genes, a new strain IPFG02 (Δ*floxA*::Km) was generated where the *floxA* gene was specifically replaced by a kanamycin resistance cassette. Strains *D. vulgaris* WT, IPFG01 and IPFG02 were grown in MOY media containing either lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), hydrogen-sulfate (HS4) or ethanol-sulfate (ES4) (Figure 4.6, Table 4.5).

Strains WT, IPFG01 and IPFG02 behaved similarly to WT on lactate conditions, both with sulfate or sulfite as terminal electron acceptors (Figure 4.6A and 4.6B and Table 4.5), whereas on pyruvate fermentation IPFG01 and IPFG02 had a slightly faster growth rate, but the final OD was slightly lower than the WT (Figure 4.6C). In contrast, in ethanol-sulfate conditions neither mutant strain, IPFG01 or IPFG02, was able to grow (Figure 4.6D and Table 4.5). In HS4 medium strains IPFG01 and IPFG02 grew somewhat slower than the WT and reached a lower cell density. To confirm that these results are due specifically to the absence of the *floxA* gene, we constructed a FloxA-complemented strain expressing FloxA from a plasmid, strain IPFG03 (IPFG02 + pMOIP12P). Strain IPFG03 grew well in ES4, with a growth rate similar to the WT, confirming that FloxA is essential for ethanol oxidation in *D. vulgaris*. Ethanol has also been reported as a metabolic product of *D. vulgaris* Hildenborough, produced in higher amounts with pyruvate as an electron donor than with lactate (Traore *et al.* 1981). To check whether the Hdr-Flox proteins are involved in the production of ethanol as a fermentative product during growth on pyruvate, we quantified ethanol accumulated in the growth media of the WT, IPFG01 and IPFG02 strains. The WT strain accumulated much higher

Table 4.5 - Specific growth rate (μ_g), doubling time (T_d) and maximal OD (600 nm) for *D. vulgaris* and mutant strains in different conditions: lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), ethanol-sulfate (ES4) and hydrogen-sulfate (HS4). IPFG01 - *hdrC*::ΩKm; IPFG02 - Δ*floxA*::Km ; IPFG03 – Δ*floxA*::Km + pMOIP12.

Medium	LS4			LS3			P			ES4			HS4		
Strain	μ_g (h^{-1})	T_d (h)	Max. OD_{500}	μ_g (h^{-1})	T_d (h)	Max. OD_{600}	μ_g (h^{-1})	T_d (h)	Max. OD_{600}	μ_g (h^{-1})	T_d (h)	Max. OD_{600}	μ_g (h^{-1})	T_d (h)	Max. OD_{600}
WT	0,174	4,0	1,257	0,315	2,2	0,715	0,207	3,3	0,464	0,119	5,8	0,627	0,040	17,4	0,715
IPFG01	0,169	4,1	1,228	0,290	2,4	0,676	0,226	3,1	0,414	0	0	0,083	0,025	28,0	0,626
IPFG02	0,172	4,0	1,184	0,300	2,3	0,689	0,219	3,2	0,391	0	0	0,078	0,026	27,0	0,633
IPFG03	0,150	4,6	1,267	0,305	2,3	0,690	0,168	4,1	0,454	0,119	5,8	0,654	0,038	18,3	0,733

levels of ethanol than the two mutant strains, both after 12h and 48h (Table 4.6), clearly pointing to a role of Flox-Hdr in ethanol production during pyruvate fermentation.

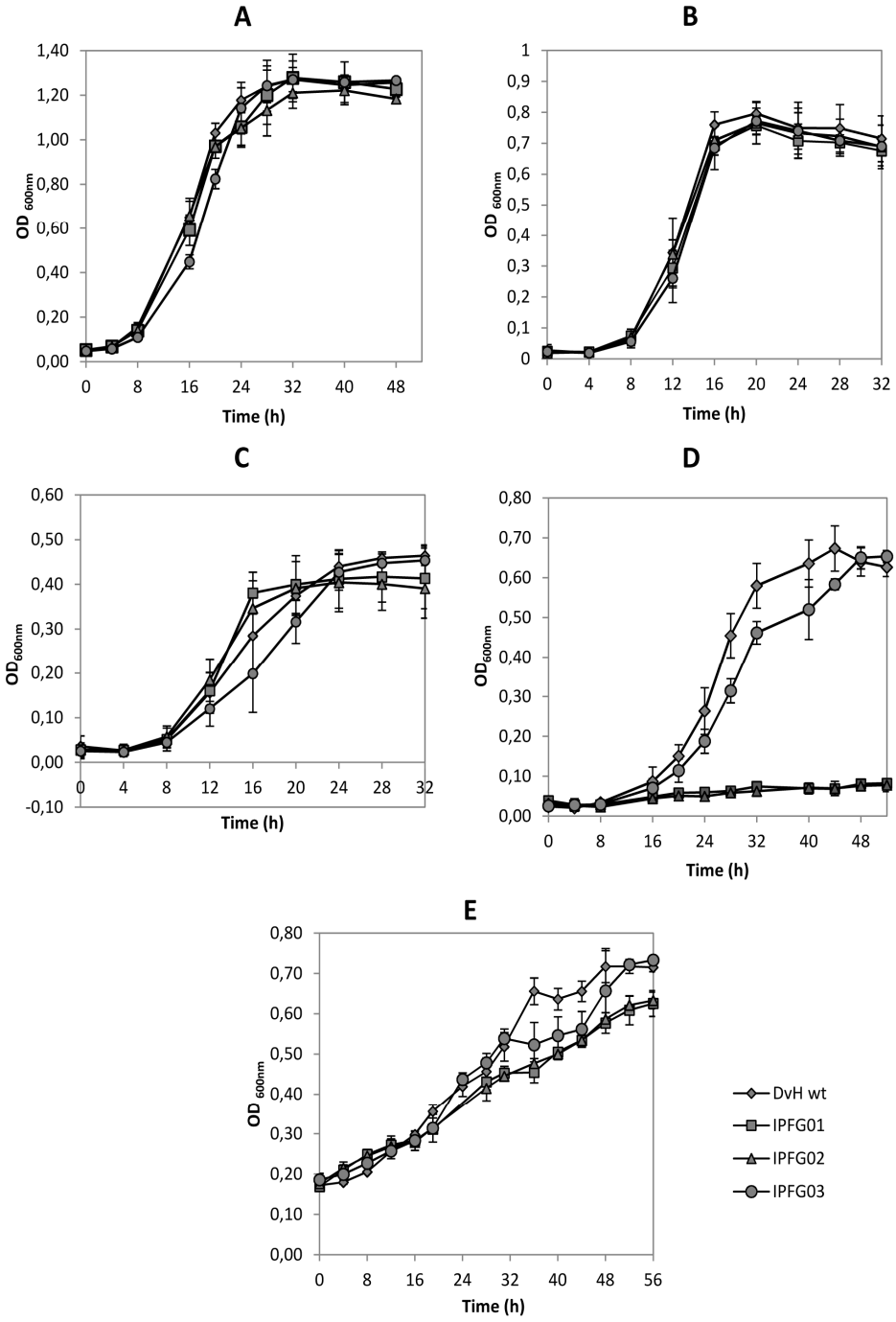

Figure 4.6 - Growth curves of *D. vulgaris* WT and mutant strains in (A) lactate-sulfate, (B) lactate-sulfite (C) pyruvate fermentation, (D) ethanol-sulfate and (E) hydrogen-

sulfate conditions. The points are means of four independent growth experiments, and error bars give standard deviations. IPFG01 - *hdrC*::ΩKm; IPFG02 - Δ*floxA::*Km; IPFG03 – Δ*floxA::*Km + pMOIP12 (complemented strain).

Table 4.6 - Ethanol quantification *D. vulgaris* WT and mutant strains, IPFG01 and IPFG02 during growth on Pyruvate (P).

Ethanol (μM)	12 h	48 h
WT	1702	1590 ± 94
IPFG01	196 ± 4	128 ± 50
IPFG02	177 ± 31	98 ± 30

4.4.6 - PROTEIN PURIFICATION

To test whether the FloxABCD-HdrABC complex is involved in electron bifurcation it is essential to isolate the proteins and perform the bifurcation assays *in vitro*. These proteins have never been isolated or characterized, so we tested several purification protocols. The purification was monitored by Western blot using antibody against FloxA and HdrA and also by enzymatic activity, following NADH oxidation activity with ferricyanide. Cells grown in ethanol have a lower growth yield and higher doubling time than those grown in lactate, and since the expression levels of Flox and Hdr proteins is still significant in LS4, we first used *D. vulgaris* WT cells grown in lactate-sulfate for anaerobic purification in two different approaches. In the first approach, the soluble crude extract was purified in two anionic exchange chromatographies and a gel filtration step in 50 mM Tris-HCl (pH 7.6)

buffer. In the second approach two anionic exchange chromatographic steps followed by hydrophobic interaction chromatography (HiTrap™ Phenyl HP) in 50 mM Phosphate buffer (pH 6.5) was used. Unfortunately, we were not successful in isolating either FloxABCD or HdrABC proteins with either of these procedures. To try to overcome the difficulty in purification we generated a strain (IPFG04) encoding a tagged version of FloxA, by fusing a Strep-tag coding sequence to the chromosomal *floxA* via double homologous recombination. Strain IPFG04 was then used to attempt purification of the Flox-Hdr complex by affinity chromatography using Strep-tactin resin. With this procedure we were able to detect by Western blot a band with the same molecular mass as FloxA in small scale purifications. Large scale purifications of IPFG04 cells grown in either LS4 or ES4 were performed with three distinct approaches: i) affinity purification (Strep-tactin resin) only; ii) anion exchange (Q-Sepharose HP) followed by affinity purification; and iii) anion exchange followed by hydrophobic interaction chromatography (HiTrap™ Phenyl HP). FloxA could be easily identified in the Western blot monitorization of the purifications, but the levels of the protein were very low, as no corresponding band could be identified by Coomassie staining in SDS-PAGE gels. Despite several attempts, we were not able to isolate the Flox or Hdr proteins, which prevented further studies into their catalytic activity, namely investigation of the FBEB mechanism. This failure seemed to be due to a very low level of Flox and Hdr proteins present in *D. vulgaris*, even in ethanol-grown cells. In contrast, we purified without difficulty the Adh1 protein in high amount to a specific activity of 4.6 U/mg, confirming that this protein is abundant in *D. vulgaris*. To check if

other *Desulfovibrio* organisms presented higher expression levels of the Flox proteins, we analyzed by Western blot the expression of FloxA in other *Desulfovibrio* spp. (*D. desulfuricans* ATCC 27774, *D. gigas* DSM1382 and *D. fructosovorans* - Supplementary Material). However, none of the tested organisms presented higher expressions levels than *D. vulgaris* in either LS4 or ES4 media.

4.5 - DISCUSSION

SRO are a group of organisms whose energy metabolism is still not completely elucidated. A genomic analysis of energy metabolism genes in SRO revealed the presence of several proteins that in other organisms have been shown to perform the new mechanism of FBEB, suggesting that this new mechanism is also important for the bioenergetics of SRO (Pereira *et al.* 2011). FBEB is a new mechanism of energy coupling that operates in anaerobes and allows the thermodynamically unfavourable reduction of ferredoxin (Buckel and Thauer 2013). Reduced ferredoxin then drives catabolic reactions requiring low redox potential, and/or its oxidation by membrane complexes such as Rnf or Ech is coupled to chemiosmotic energy conversion. One example of a protein that performs FBEB is the HdrABC heterodisulfide reductase of hydrogenotrophic methanogens. HdrABC forms a complex with the MvhADG hydrogenase (Thauer *et al.* 2008; Kaster *et al.* 2011) or with the FdhAB formate dehydrogenase (Costa *et al.* 2010), coupling the reduction of the CoM-S-S-CoB heterodisulfide with H_2 or formate to the reduction of ferredoxin (Figure 4.7A). The protein believed to perform the bifurcation of electrons is the flavoprotein HdrA. HdrA and HdrABC

proteins are found in many SRO, even though these organisms do not contain the CoM-S-S-CoB heterodisulfide (Pereira *et al.* 2011). In particular, a new gene cluster *hdrCBA-floxCDBA* was found to be present in many bacterial SRO, and proposed to encode a new putative NAD(P)H dehydrogenase (FloxABCD) forming a complex with HdrABC analogous to the MvhADG-HdrABC complex of methanogens (Figure 4.7) (Pereira *et al.* 2011).

In the past few years several genomic, transcriptomic and proteomic methods have been used to study the energy metabolism of SRO, using the model organism *D. vulgaris* Hildenborough and other *Desulfovibrio* species (Haveman *et al.* 2003; Zhang *et al.* 2006a; Zhang *et al.* 2006b; Walker *et al.* 2009; Meyer *et al.* 2013). The *flox-hdr* genes are often referred in these studies, suggesting a relevant function in the energy metabolism, although not essential for sulfate reduction. In *D. vulgaris*, the *flox-hdr* gene cluster was first identified next to the gene for an alcohol dehydrogenase *adh1* (DVU2405), which is a highly expressed protein in *D. vulgaris* grown with lactate, pyruvate, formate, ethanol or hydrogen as electron donors for sulfate reduction (Haveman *et al.* 2003). The expression of the *adh1* gene was upregulated in ethanol grown cells, and a mutant lacking this gene was unable to grow in ethanol-sulfate medium (Haveman *et al.* 2003), indicating that Adh (DVU2405) is the main enzyme catalyzing ethanol oxidation in *D. vulgaris*. The *flox-hdr* genes were proposed to code for a hydrogenase:heterodisulfide reductase complex involved in a hydrogen cycling mechanism (Haveman *et al.* 2003), but the *flox* genes do not encode a hydrogenase.

In this work, we investigated further the physiological function of the *flox-hdr* genes in *D. vulgaris*. We constructed a mutant strain IPFG01, where a ΩKm cassette leads to premature termination of the transcription of *hdrC* and the downstream genes. The presence of FloxA could not be detected in this strain, confirming the predictions that the *hdrABC* and *floxABCD* genes are part of the same transcriptional unit. Gene and protein expression studies of *D. vulgaris* cells grown with different electron donors for sulfate or sulfite reduction showed that the highest expression of the *floxA* and *hdrA* genes is observed with ethanol as electron donor. Furthermore, in agreement with previous reports (Haveman *et al.* 2003), the *adh1* gene is much more expressed than *floxA* and *hdrA*, further indicating that *adh1* is not part of the same operon as the *flox-hdr* genes. This difference in gene expression was also reflected in the protein purification. Although we could purify Adh1 without difficulty, we were not successful in isolating the Flox or Hdr proteins. FloxA could be easily identified by Western blot in protein purification fractions, but no corresponding band would be present in Coomassie stain of SDS-PAGE gels, which meant that the levels of FloxA present were very low. We concluded that the transcription levels of the Flox-Hdr proteins in *D. vulgaris* are very low, which agrees with previous microarray results (Haveman *et al.* 2003). It is also possible that these proteins are unstable, preventing their isolation. Despite the generation of strain IPFG04, with a tagged FloxA we were not able to isolate the Flox-Hdr proteins in sufficient amounts for further biochemical studies, even using cells grown in ethanol.

Phenotypic studies of strains IPFG01 and IPFG02, revealed that both strains are not able to grow using ethanol as electron donor for sulfate reduction. In the complemented strain IPFG03, growth with ethanol was restored confirming that the Flox-Hdr proteins and FloxA individually, are essential in the metabolic pathway for ethanol oxidation, which agrees with their higher expression in this condition. The probable pathway involving Adh1 and Flox-Hdr proteins starts with oxidation of ethanol by Adh1 with reduction of NAD^+ to NADH (Figure 4.7B). FloxA, which has a FAD and a NAD(P) binding domain, oxidizes NADH and electrons are transferred to FloxB and FloxCD. FloxCD transfers electrons to HdrABC, probably through the MvhD-like domain in FloxCD, similarly to what happens in methanogens. The electron acceptor of HdrABC is still a question mark. As suggested before (Pereira *et al.* 2011), we propose that this electron acceptor is the cysteine containing protein DsrC (Venceslau *et al.* 2014). In support of this hypothesis the *dsrC* gene is found next to a *hdrA/L-floxACBD* gene cluster in some SRO, including *Desulfobacterium autotrophicum* (Figure 4.2), *Desulfosarcina* sp. BUS5 and *Desulfatirhabdium butyrativorans* (Venceslau *et al.* 2014). DsrC is one of the electron donors to the DsrAB sulfite reductase (Oliveira *et al.* 2008), and in sulfate reducing organisms is one of the most highly abundant energy metabolism proteins (Haveman *et al.* 2003; Wall *et al.* 2008; Keller and Wall 2011). Since the reduction of ferredoxin by NADH is an unfavourable reaction, and by analogy to the function of HdrABC in methanogens, we propose that the oxidation of NADH by FloxABCD involves the process of FBEB, coupling the endergonic reduction of ferredoxin by NADH to the (presumably) exergonic reduction of DsrC by

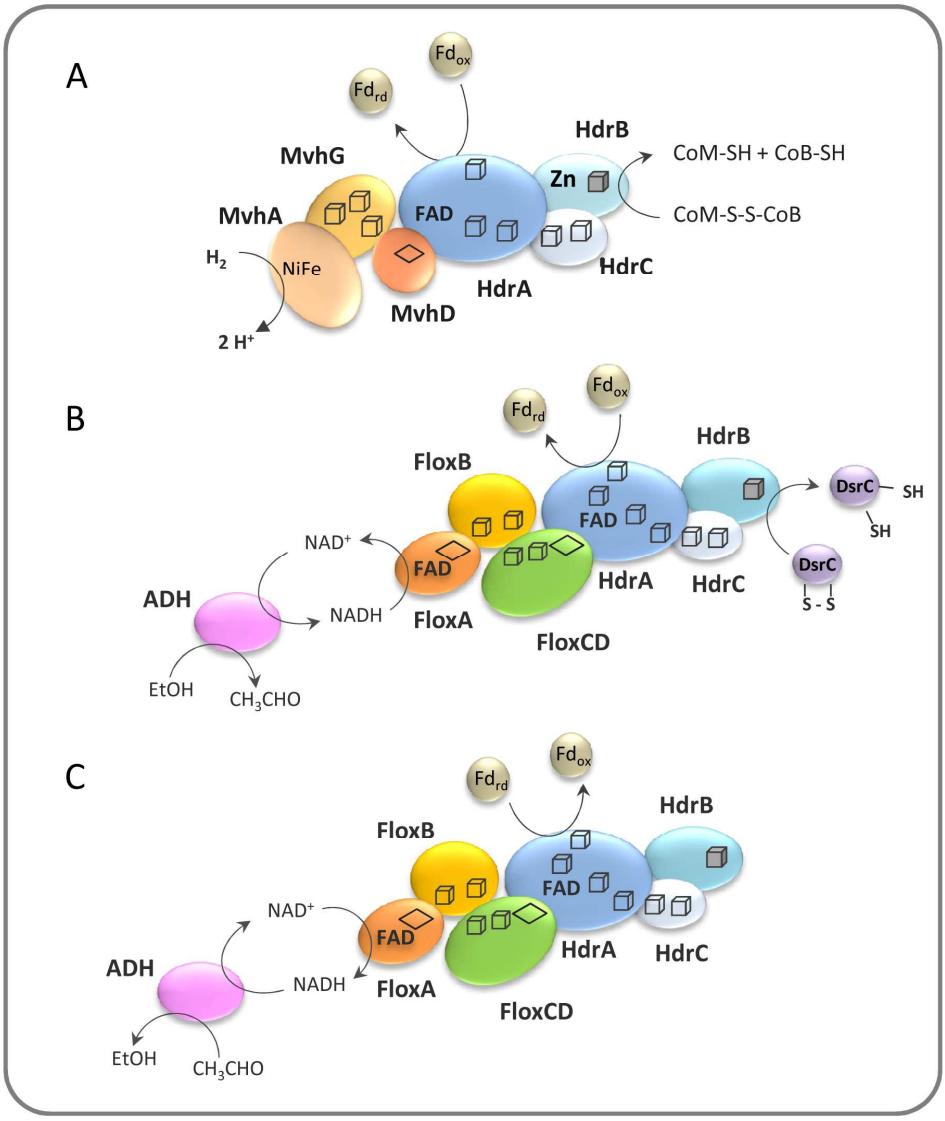

Figure 4.7 - Function of the HdrABC-MvhADG and HdrABC-FloxABCD complexes. (A) In the last step of methanogenesis the MvhADG hydrogenase - HdrABC complex uses H_2 to reduce both the heterodisulfide and Fd_{ox} using the FBEB mechanism (Kaster *et al.* 2011). (B) In *D. vulgaris* growing on ethanol/sulfate, Adh oxidizes ethanol producing NADH that is oxidized by FloxABCD. Electrons are transferred to HdrABC, which can then bifurcate them to Fd and a second electron acceptor that we propose is DsrC. (C) In *D. vulgaris* growing by pyruvate fermentation, Adh reduces acetaldehyde to ethanol with NADH. The NAD^+ is recycled by FloxABCD, with electrons coming from Fd_{red} through HdrABC.

NADH (Figure 4.7B). Regeneration of NADH involving FloxABCD-HdrABC is essential for the bioenergetics of *D. vulgaris* when it is growing on ethanol/sulfate, but not when the cells grow with lactate/sulfate or pyruvate/sulfate, which agrees with the idea that pyridine nucleotides are not directly involved in the oxidation of lactate or pyruvate.

A recent study proposed a function for the *hdr-flox* genes in *Desulfovibrio alaskensis* G20, which has two *hdr-flox* operons (*hdr-flox*-1 and *adh-hdr-flox*-2), one of them containing an *adh* gene next to the operon (*adh-hdr-flox*-2), as in *D. vulgaris.* In *D. alaskensis* G20, gene fitness studies of a transposon mutant library indicated that mutants in the *hdr-flox1* genes (but not in the *adh-hdr-flox2* genes) had a reduced fitness relative to the wild type in growth by pyruvate fermentation, syntrophic growth on pyruvate with a methanogen and pyruvate/sulfate respiration (Meyer *et al.* 2014). Although the expression of the *hdr-flox1* genes was not increased in these conditions, phenotypic studies with individual mutants of these genes revealed also slightly lower growth rates and/or final optical densities than the wild type. These results pointed to the involvement of Hdr-Flox1 in pyruvate metabolism by *D. alaskensis* G20 both in fermentation/syntrophy and in respiration, although not in an essential role. This agrees with our observations of reduced final optical densities for the IPFG01 and IPFG02 mutant strains grown by pyruvate fermentation.

Meyer *et al.* proposed that in *D. alaskensis* G20 the Hdr-Flox1 proteins are responsible for NADH oxidation in a pathway leading to succinate production (Meyer *et al.* 2014). They proposed that ferredoxin reduced upon pyruvate oxidation to acetyl-CoA is oxidized by the membrane Rnf

complex, which can either: i) transfer electrons for the periplasmic TpIc_3, leading to H_2 or formate production (suggested as the most significant pathway during syntrophic co-culture), or ii) produce NADH that is used by Hdr-Flox1 to re-reduce ferredoxin and DsrC (suggested as a more relevant pathway during pyruvate fermentation). They propose that reduced DsrC is then oxidized by HmcEF, reducing menaquinone, which serves as the electron donor for fumarate reduction to succinate by a membrane-associated fumarate reductase (Meyer *et al.* 2014). However, this proposal is not consistent with the shift in fermentation products reported therein for the mutants, as the *hdrA*, *hdrB*, *floxA* and *floxC* deletion mutants produce more succinate and significantly less H_2 than the wild type strain (Meyer *et al.* 2014), which is the opposite of what would be expected according to this proposal (less succinate due to interruption of the proposed pathway for menaquinone reduction involving Hdr-Flox1, and more H_2 from the Hdr-Flox1-independent pathway to compensate for that). Ethanol production was not measured in these experiments. We reasoned that Hdr-Flox1 could instead be involved in recycling NAD^+ produced during Adh reduction of acetaldehyde to ethanol, formed as an alternative pyruvate fermentation product (Figure 4.7C). Production of ethanol from pyruvate as carbon and energy source has been reported before for *D. vulgaris* (Traore *et al.* 1981). This idea is supported by the reported strong upregulation of the aldehyde:ferredoxin oxidoreductase in *D. alaskensis* G20, in both syntrophic and fermentative conditions with pyruvate (Meyer *et al.* 2014). In addition, in an earlier study of syntrophic growth of *D. vulgaris* Hildenborough with a methanogen on lactate, upregulation

of the genes coding for Aor and Adh1-Hdr-Flox was also observed, and ethanol was reported as a by-product during syntrophic growth (Walker *et al.* 2009). We measured ethanol production from *D. vulgaris* cells growing by pyruvate fermentation, which showed that the IPFG01 and IPFG02 mutants produced reduced levels of ethanol, compared to the WT strain (Table 4.6). These results confirm that the role of the Flox-Hdr proteins in fermentative conditions is in the production of ethanol, and thus in recycling NAD^+ (Figure 4.7C), and not in NADH oxidation as proposed by Meyer *et al.*. The reduction of NAD^+ will be performed by FloxA with electrons coming from ferredoxin oxidation by HdrABC. This reaction is favourable and does not require FBEB. Ferredoxin is reduced by the pyruvate:ferredoxin oxidoreductase (Figure 4.8).

Coming back to the study of pyruvate metabolism in *D. alaskensis* G20, the shift in fermentation products for the *hdr-flox1* mutants (Meyer *et al.* 2014) points, not to a role in a putative fumarate reduction pathway, but instead to an involvement of Hdr-Flox1 in a H_2 production pathway, although in what role is not clear, and we would rather not speculate. Interestingly, Haveman and co-workers reported on a reduced expression of the *adh1-hdr-flox* genes in a *D. vulgaris* Hildenborouh mutant strain lacking the *hydAB* genes that code for the periplasmic [FeFe] hydrogenase (Haveman *et al.* 2003), also linking the Adh1-Hdr-Flox proteins to periplasmic H_2 metabolism. In our work, there was a slight impairment of growth on hydrogen-sulfate for the IPFG01 and IPFG02 strains, showing a slightly reduced growth rate, which also agrees with the involvement of these proteins in H_2 metabolism.

Recently, a homologous *hdr-flox* gene cluster present in the genome of the syntrophic organism *Syntrophorhabdus aromaticivorans* (SynarDRAFT_0715-0709) was proposed by Nobu *et al.* to encode a putative ion-translocating ferredoxin:NADH oxidoreductase, corresponding to the *flox* genes, that they named Ifo (Nobu *et al.* 2014). They suggest that Ifo is a membrane-associated ion-translocating complex that uses reverse electron transport to achieve ferredoxin reduction from NADH, coupled to oxidation of DsrC by HdrABC. This proposal is based solely on the assignment of a β-barrel domain in Synardraft_0709 (corresponding to *floxA*) as a transmembrane region capable of ion-translocation. We analyzed the sequence of this subunit and of other homologous *floxA* genes using several secondary structure prediction algorithms, including the one used by these authors (SABLE), and none predicted a transmembrane association for this C-terminal β-barrel domain. PRED-TMBB, a specific web server for predicting the topology of β-barrel membrane proteins, also did not predict a membrane-association for this domain. In fact, this domain includes seven β-sheets that form a Greek Key barrel arrangement, similar to the β-barrel domain of riboflavin synthase (IPR017938) and other FAD-binding oxidoreductases, all of which are entirely soluble proteins. Thus, the proposal of a new ion-translocating complex has no real support and the *S. aromaticivorans* SynarDRAFT_0715-0709 genes actually encode a soluble HdrABC-FloxABCD complex very similar to that of *D. vulgaris* (Figure 4.2). Interestingly, the *S. aromaticivorans* genome includes a *dsrC* gene (also proposed to be the physiological partner of HdrABC), but no *dsrAB* genes. Further biochemical studies will be required to establish if

this gene is actually a DsrC protein, or if it is a closely related TusE protein that functions in the biosynthesis of 2-thiouridine (Venceslau *et al.* 2014).

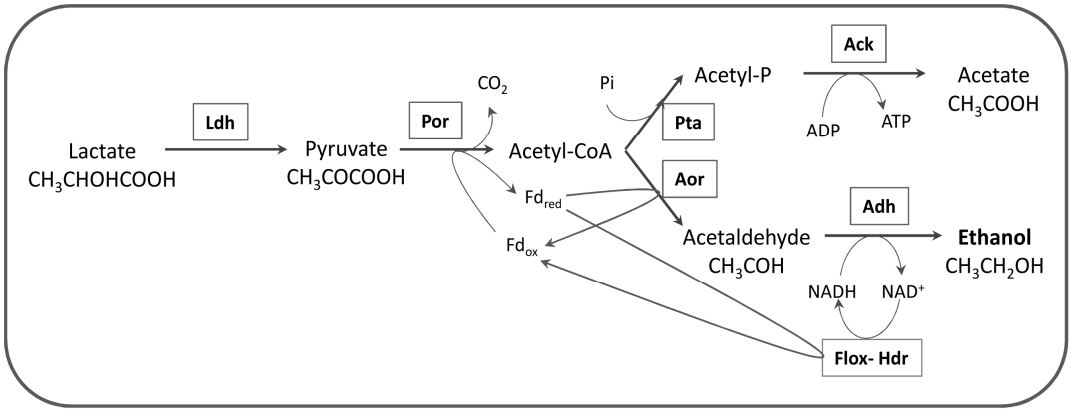

Figure 4.8 - Pathway of fermentative lactate and pyruvate oxidation in *Desulfovibrio* spp. Acetyl-CoA is mainly converted to acetyl-phosphate and this to acetate, producing ATP by substrate-level phosphorylation. In a parallel pathway, acetyl-CoA may also be reduced by aldehyde:ferredoxin oxidoreductase (Traore *et al.* 1981) to acetaldehyde, which is then converted to ethanol by alcohol dehydrogenase (Adh). This pathway will be more relevant in fermentative conditions. Abbreviations: Ldh, lactate dehydrogenase; Por, pyruvate:ferredoxin oxidoreductase; Pta, phosphate acetyltransferase; Ack, acetate kinase; Aor, aldehyde:ferredoxin oxidoreductase.

In conclusion, we showed that the recently recognized FloxABCD proteins are essential for ethanol oxidation in *D. vulgaris* and are co-expressed with the HdrABC proteins, forming a complex that we propose to perform the coupled reduction of ferredoxin and a disulfide (the DsrC protein in SRO) using the FBEB mechanism. In pyruvate fermentation, the FloxABCD-HdrABC complex operates in reverse to reduce NAD^+, allowing the production of ethanol and the regeneration of oxidized

ferredoxin. It is clear that the FloxABCD-HdrABC proteins are involved in the metabolism of pyridine nucleotides, and thus this work contributes to a better understanding of the role of these cofactors in SRO, which has been poorly defined so far. Importantly, it provides the first link between these cofactors and sulfate (sulfite) reduction through the DsrC protein. In a broader perspective, it is also important to note that the *hdr-flox* gene cluster is not exclusive of SRO, but is widespread among many anaerobic bacteria, and thus FloxABCD constitutes a novel family of NAD(P)H oxidoreductases. The presence of the *hdr-flox* gene cluster in many different Bacteria, and the strictly conserved organization of the genes reveal that the so far unidentified FloxABCD proteins, together with HdrABC, perform a general and important function in the energy metabolism of these organisms. As discussed above, the function of this protein complex will be to oxidize NADH with reduction of ferredoxin and a high-redox-potential disulfide acceptor, involving the FBEB mechanism. Thus, this work identifies a new set of proteins possibly involved in FBEB, further demonstrating the importance of this mechanism for the bioenergetics of anaerobic prokaryotes (Buckel and Thauer 2013). Future work will focus on trying to isolate this complex from other organisms to confirm *in vitro* the FBEB process.

4.6 - Acknowledgements

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4.7 – SUPPLEMENTARY MATERIAL

Supplementary material can be found in Appendix II.

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CHAPTER 5

CONCLUDING REMARKS

A new mechanism for energy coupling has been proposed in the last few years, the Flavin Based Electron Bifurcation (FBEB). This mechanism allows the coupling of thermodynamically unfavorable reactions to favorable ones. The FBEB mechanism has been demonstrated experimentally in acetogens, methanogens and fermentative organisms, and it is believed to have been present in the early stages of life as a primitive mechanism to obtain energy. Several proteins involved in FBEB seem to be widespread in prokaryotes, suggesting that electron bifurcation is a general mechanism for conserving energy in chemotrophic anaerobic bacteria. In one of these groups of prokaryotes, sulfate reducers, we can find the incidence of **H**etero**d**isulfide **r**eductase (Hdr)-related proteins, in particular HdrA-like subunits. HdrA is involved in FBEB by an HdrABC/MvhADG complex in hydrogenotrophic methanogens, in which the endergonic reduction of ferredoxin (Fd) by H_2 is coupled to the exergonic reduction of the CoM-S-S-CoB heterodisulfide by H_2. The presence of these flavin containing subunits in sulfate reducers suggests that FBEB can constitute alternative pathways for energy conservation in sulfate reducing organisms (SRO).

In our genomic analysis a gene locus containing *sat*, *aprBA* and *qmoABC* genes is present in the majority of SRO analyzed, except *Caldivirga maquilingensis* that lack the *qmo* genes and the Gram-positive bacteria that lacks the *qmoC* gene. A direct connection between QmoABC and sulfate reduction was established when a *Desulfovibrio vulgaris* Hildenborough mutant lacking the *qmoABC* genes was not able to grow with sulfate, but grew well with sulfite or thiosulfate as electron donor. This evidence demonstrated that the Qmo complex is involved in

electron flow between the menaquinone pool and adenosine 5'-phosphosulfate (APS) reduction. However, direct electron transfer between the Qmo complex and Apr could not be detected, which could suggest the involvement of third partners in the pathway.

This work provided for the first time direct evidence that QmoABC interacts with AprBA in *Desulfovibrio* spp. *in vitro* and *in vivo*. However, no direct electron transfer was observed which led us to propose a reverse electron bifurcation, i.e. electron confurcation. The electron confurcation mechanism proposed considers that menaquinol and a cytoplasmic reductant with low redox potential (probably Fd) could both donate electrons to the Qmo complex, which would confurcate electrons to the APS reductase and reduce APS. Thus coupling APS reduction with menaquinone pool oxidation through electron confurcation, contributes to the chemiosmotic energy conservation during sulfate reduction. For future experiments we propose the reconstitution of the system into lipossomes to study the possible electron transfer between Qmo and Apr. In addition to the Qmo complex we suggest the inclusion of the **Q**uinone-**r**eductase **c**omplex (QrcABCD) for the menaquinone pool recycling. The Qrc complex is involved in electron transfer from the periplasm (from hydrogenases and formate dehydrogenases) through Type I cytochrome c_3 (TpIc_3), which are transferred to the menaquinone pool. Therefore, the redox loop generated with Qrc and Qmo can contribute to the proton motive force (pmf) during sulfate reduction with H_2 or formate as electron donors. The only obstacle to these experiments is the absence of menaquinol oxidation activity associated

to Qmo complex, thus further electron acceptors have to be tested so that a reliable assay can be achieved.

Besides HdrA-homologous proteins, we can find in sulfate reducers an *hdrA* gene in two types of gene loci. In the first type an *hdrA* gene or a set of *hdrABC* genes are found next to *mvhDGA* genes coding for a soluble F_{420}-non-reducing hydrogenase (Mvh). In the second type, again a single *hdrA* gene or a set of *hdrABC* genes are found next to four genes that we named *floxABCD* genes (for ***fl***avin ***ox***idoreductase). The FloxABCD is a new NADH dehydrogenase complex that was initially identified as a putative hydrogenase (Hase), as the *flox* genes are annotated as putative Hase-related to subunits of *Pyrococcus furiosus* NAD(P)-dependent soluble Hases I and II. The lack of a gene coding for a catalytic Hase subunit made us reclassify the protein as a new NADH dehydrogenase involved in NAD(P)H oxidation and electron transfer to HdrABC proteins probably through FBEB.

The *floxABCD-hdrABC* genes are often implicated in gene expression and proteomic studies of *D. vulgaris* energy metabolism and in syntrophic growth studies with methanogens, thus these genes should play an important role in the energy metabolism of this deltaproteobacterium. In our genomic analysis the *floxABCD-hdrA* or *floxABCD-hdrABC* genes are present in the majority of the SRO analyzed and they can also be found in a large number of Bacteria including members of many different phyla such as *Chlorobi, Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria, Spirochaetes, Actinobacteria* and *Acidobacteria*. The *flox-hdr* gene cluster organization is strictly conserved among the different

Bacteria which points to a general and important function of these genes in the energy metabolism of anaerobes.

We observed that the Flox-Hdr proteins are essential to ethanol metabolism in a pathway involving Adh1. In respiratory conditions the pathway most likely starts with ethanol oxidation by Adh1 generating NADH that is oxidized by FloxABCD. Electrons are then transferred to HdrABC, which can bifurcate them to Fd and to a second electron acceptor that we propose to be DsrC. In fermentative conditions the proteins seems to work in the opposite direction, and NAD^+ is reduced to NADH by FloxA with electrons coming from Fd oxidation by HdrABC, while NADH is oxidized by Adh1 and ethanol is formed. Therefore, this work demonstrated that in *D. vulgaris*, the FloxABCD-HdrABC proteins are essential for ethanol metabolism and are also involved in pyridine nucleotides metabolism in *Desulfovibrio* spp., probably involving a FBEB mechanism. Future experiments involving protein isolation and *in vitro* study of FBEB is necessary to confirm the mechanism proposed.

In summary, the work presented in this thesis demonstrates the importance of FBEB mechanism for the bioenergetics of anaerobic prokaryotes, in particular to SRO. The genomic analysis in SRO also support these findings and a high number of cytoplasmic hydrogenases, formate dehydrogenases and heterodisulfide reductase-like proteins suggests that electron bifurcation mechanisms may be involved in energy coupling in sulfate reducers. Nevertheless, further work is necessary to elucidate the mechanisms of energy conservation involved in sulfate reduction.

APPENDICES

APPENDIX I

SUPPLEMENTARY MATERIAL OF CHAPTER 2

Table I.1 – Analysis of *nfnAB* gene distribution in the SRO genomes.

	NfnA	NfnB
Archaea		
Archaeoglobus fulgidus		
Archaeoglobus profundus		
Caldivirga maquilingensis		
Deltaproteobacteria		
Desulfovibrionacae		
Desulfovibrio aespoeensis		
Desulfovibrio desulfuricans G20	1	1
Desulfovibrio desulfuricans ATCC 27774	1	1
Desulfovibrio magneticus RS-1	1	1
Desulfovibrio piger	1	1
Desulfovibrio salexigens	1	1
Desulfovibrio sp. FW1012B	1	1
Desulfovibrio vulgaris Hildenborough	1	1
Desulfomicrobiacae		
Desulfomicrobium baculatum	1	1
Desulfobacteraceae		
Desulfatibacillum alkenivorans	1	1
Desulfobacterium autotrophicum HRM2	1	1
Desulfococcus oleovorans Hxd3	1	1
Desulfohalobiacae		
Desulfohalobium retbaense DSM 5692	1	1
Desulfonatronospira thiodismutans ASO3-1	1	1
Desulfobulbaceae		
Desulfotalea psychrophila		
Desulfurivibrio alkaliphilus	1	1
Syntrophobacteraceae		
Syntrophobacter fumaroxidans MPOB	1	1
Clostridia		
Peptococcaceae		
Desulfotomaculum acetoxidans DSM 771	1	1
Desulfotomaculum reducens	1	1
C. Desulforudis audaxviator MP104C	1	1
Thermoanaerobacterales		
Ammonifex degensii KC4	1	1
Nitrospirae		
Thermodesulfovibrio yellowstonii		
Nº of organisms	19	19

GENE LOCUS

	Locus	Sat	AprA	QmoA	DsrA	DsrC	DsrK
Archaea							
Archaeoglobus fulgidus	AF	1667	1670	0663	0423	2228	0502; '0547; '0543
Archaeoglobus profundus	Arcpr_	1264	1261	1260	0139	1726	1730
Caldivirga maquilingensis	Cmaq_	0274	0273		0853	0856	0851
Deltaproteobacteria							
Desulfovibrionacae							
Desulfovibrio aespoaensis	DaesDRAFT_	2031	2029	2028	2438	0796	0513
Desulfovibrio desulfuricans G20	Dde_	2265	1110	1111	0526	0762	2272
Desulfovibrio desulfuricans ATCC 27774	Ddes_	0454	2129	2127	2275	1917	0450
Desulfovibrio magneticus RS-1	DMR_	39470	05400	05410	03600	15890	03710
Desulfovibrio piger	DESPIG_	02241	02773	02771		02353	02236
Desulfovibrio salexigens	Desal_	0228	0230	0231	0787	0984	0185
Desulfovibrio sp. FW1012B	DFW101DRAFT_	0832	1162	1163	3451	2958	3642
Desulfovibrio vulgaris Hildenborough	DVU	1295	0847	0848	0402	2776	1289
Desulfomicrobiacae							
Desulfomicrobium baculatum	Dbac_	3196	3198	3199	0279	2958	0271
Desulfobacteraceae							
Desulfatibacillum alkenivorans	Dalk_	2445	1569	1568	4301	4140	0606
Desulfobacterium autotrophicum HRM2	HRM2_	31180	04510	04500	42400	22050	31650
Desulfococcus oleovorans Hxd3	Dole_	1002	0998	0999	2669	0463	0451
Desulfohalobiacae							
Desulfohalobium retbaense DSM 5692	Dret_	1968	1966	1965	0244	1739	0236
Desulfonatronospira thiodismutans ASO3-1	DthioDRAFT_	1410	1407	1406	2272	2675	2263
Desulfobulbaceae							
Desulfotalea psychrophila	DP	1472	1105	1106	0797	0997	3074
Desulfurivibrio alkaliphilus	DaAHT2_	0293	1471	1470	0296	2041	2299
Syntrophobacteraceae							
Syntrophobacter fumaroxidans MPOB	Sfum_	1046	1048	1287	4042	4045	1147
Clostridia							
Peptococcaceae							
Desulfotomaculum acetoxidans DSM 771	Dtox_	3579		3576	0079	0077	0076
Desulfotomaculum reducens	Dred_	0635	3577	0638	3187	3197	0635
C. Desulforudis audaxviator MP104C	Daud_	1076	0637	1884	2201	2190	2191
Thermoanaerobacterales							
Ammonifex degensii KC4	Adeg_	1712	1080	1079	2094	0035	0034
Nitrospirae							
Thermodesulfovibrio yellowstonii	THEYE_	A1835	A1832	A1831	A1994	A0003	A0006

	Locus	QrcA	TmcA	HmcA	NhcB	Cyt c_3
Archaea						
Archaeoglobus fulgidus	AF					
Archaeoglobus profundus	Arcpr_					
Caldivirga maquilingensis	Cmaq_					
Deltaproteobacteria						
Desulfovibrionacae						
Desulfovibrio aespoeensis	DaesDRAFT_	1214	1365	1617	1137	1104; 2527
Desulfovibrio desulfuricans G20	Dde_	2932	3710	0653		3182
Desulfovibrio desulfuricans ATCC 27774	Ddes_		0847		2039	2013
Desulfovibrio magneticus RS-1	DMR_	18010	42490	12830		02560; 21540
Desulfovibrio piger	DESPIG_				01442	02928
Desulfovibrio salexigens	Desal_	1042	3040	1378		0756; 1385; 2447
Desulfovibrio sp. FW1012B	DFW101DRAFT_	2229	1322	1417		0515; 1530
Desulfovibrio vulgaris Hildenborough	DVU	0695*	0263	0536		3171
Desulfomicrobiacae						
Desulfomicrobium baculatum	Dbac_	3390	0567	0568		1057; 3004
Desulfobacteraceae						
Desulfatibacillum alkenivorans	Dalk_	1267			1818	2817; 4187
Desulfobacterium autotrophicum HRM2	HRM2_	18930; 15120	25810; 44980			42360
Desulfococcus oleovorans Hxd3	Dole_	2546	0305; 0462	2250		3170; 0267
Desulfohalobiacae						
Desulfohalobium retbaense DSM 5692	Dret_	0270	1027	0877		0413; 1266; 1800; 2420
Desulfonatronospira thiodismutans ASO3-1	DthioDRAFT_		1590; 2239		1143	1352; 2183; 2529
Desulfobulbaceae						
Desulfotalea psychrophila	DP					
Desulfurivibrio alkaliphilus	DaAHT2_					
Syntrophobacteraceae						
Syntrophobacter fumaroxidans MPOB	Sfum_	0611				4047
Clostridia						
Peptococcaceae						
Desulfotomaculum acetoxidans DSM 771	Dtox_					
Desulfotomaculum reducens	Dred_					
C. Desulforudis audaxviator MP104C	Daud_					
Thermoanaerobacterales						
Ammonifex degensii KC4	Adeg_					
Nitrospirae						
Thermodesulfovibrio yellowstonii	THEYE_			A1283		A1056

		Cytoplasmic [NiFe]							Cytoplasmic [FeFe]			
		Soluble					Memb		Soluble			
	Locus	HdrA-Mvh	HdrABC-Mvh	Mvh	Hox	Sensor	Ech	Coo	$[FeFe]_{Bif}$	$[FeFe]_{mon}$	FHL	HsfB
Archaea												
Archaeoglobus fulgidus	AF		1372									
Archaeoglobus profundus	Arcpr_		1550									
Caldivirga maquilingensis	Cmaq_											
Deltaproteobacteria												
Desulfovibrionacae												
Desulfovibrio aespoeensis	DaesDRAFT_						0766					
Desulfovibrio desulfuricans G20	Dde_									0725	0475	
Desulfovibrio desulfuricans ATCC 27774	Ddes_						1668	1885				
Desulfovibrio magneticus RS-1	DMR_						02730		02480; 07830		43510	03530
Desulfovibrio piger	DESPIG_							02339		03092		
Desulfovibrio salexigens	Desal_						3233A				0363	
Desulfovibrio sp. FW1012B	DFW101DRAFT_						3678		2922			3457
Desulfovibrio vulgaris Hildenborough	DVU						029A	2291	1771			
Desulfomicrobiacae												
Desulfomicrobium baculatum	Dbac_											
Desulfobacteraceae												
Desulfatibacillum alkenivorans	Dalk_	4962				2281						
Desulfobacterium autotrophicum HRM2	HRM2_	11740							26580		01590	16550
Desulfococcus oleovorans Hxd3	Dole_											
Desulfohalobiacae												
Desulfohalobium retbaense DSM 5692	Dret_	2258										
Desulfonatronospira thiodismutans ASO3-1	DthioDRAFT_	0067	1312			2286						
Desulfobulbaceae												
Desulfotalea psychrophila	DP	1013			2212					2379	0479	
Desulfurivibrio alkaliphilus	DaAHT2_			1419	1419							
Syntrophobacteraceae												
Syntrophobacter fumaroxidans MPOB	Sfum_	3954; 3537		2221	2716				0844	0848		1846
Clostridia												
Peptococcaceae												
Desulfotomaculum acetoxidans DSM 771	Dtox_								0172	1708		0178
Desulfotomaculum reducens	Dred_								1651; 1654; 3290	1794; 1440		1585
C. Desulforudis audaxviator MP104C	Daud_			1651			1098A		1337	0413; 0152; 0154		
Thermoanaerobacterales												
Ammonifex degensii KC4	Adeg_			2013						1781; 1783		
Nitrospirae												
Thermodesulfovibrio yellowstonii	THEYE_		A1365	A1854						A1726;A1727		

		Hdr/Mvh		Hdr/Flox		HdrA/ other		
	Locus	HdrABC/Mvh	HdrA/Mvh	HdrABC/Flox	HdrA/Flox	HdrAL/Fdh	HdrA/Fdh	HdrAL/POR
Archaea								
Archaeoglobus fulgidus	AF	1377						
Archaeoglobus profundus	Arcpr_	1555						
Caldivirga maquilingensis	Cmaq_							
Deltaproteobacteria								
Desulfovibrionacae								
Desulfovibrio aespoeensis	DaesDRAFT_					1130		
Desulfovibrio desulfuricans G20	Dde_			3526; 1209				
Desulfovibrio desulfuricans ATCC 27774	Ddes_			2100				
Desulfovibrio magneticus RS-1	DMR_			01920				
Desulfovibrio piger	DESPIG_			02731				
Desulfovibrio salexigens	Desal_			0600				
Desulfovibrio sp. FW1012B	DFW101DRAFT_			2860				
Desulfovibrio vulgaris Hildenborcough	DVU			2402				
Desulfomicrobiacae								
Desulfomicrobium baculatum	Dbac_			0850		2345		
Desulfobacteraceae								
Desulfatibacillum alkenivorans	Dalk_		4966		5064			1624
Desulfobacterium autotrophicum HRM2	HRM2_		11710		22100	22750	11850; 14330; 14340	
Desulfococcus oleovorans Hxd3	Dole_							
Desulfohalobiacae								
Desulfohalobium retbaense DSM 5692	Dret_		2260	0196; 2315		0418		
Desulfonatronospira thiodismutans ASO3-1	DthioDRAFT_	1307	0070	0763				
Desulfobulbaceae								
Desulfotalea psychrophila	DP		1010				0822	
Desulfurivibrio alkaliphilus	DaAHT2_							
Syntrophobacteraceae								
Syntrophobacter fumaroxidans MPOB	Sfum_		3958; 3534		1977; 1974	0821		0012
Clostridia								
Peptococcaceae								
Desulfotomaculum acetoxidans DSM 771	Dtox_			1353; 1350	1277	1353; 1350		
Desulfotomaculum reducens	Dred_				0143; 0137	1328		
C. Desulforudis audaxviator MP104C	Daud_			0565				
Thermoanaerobacterales								
Ammonifex degensii KC4	Adeg_			1333				
Nitrospirae								
Thermodesulfovibrio yellowstonii	THEYE_	A1371						

		NfnA
	Locus	
Archaea		
Archaeoglobus fulgidus	AF	
Archaeoglobus profundus	Arcpr_	
Caldivirga maquilingensis	Cmaq_	
Deltaproteobacteria		
Desulfovibrionacae		
Desulfovibrio aespoeensis	DaesDRAFT_	
Desulfovibrio desulfuricans G20	Dde_	3636
Desulfovibrio desulfuricans ATCC 27774	Ddes_	1983
Desulfovibrio magneticus RS-1	DMR_	34320
Desulfovibrio piger	DESPIG_	02407
Desulfovibrio salexigens	Desal_	3492
Desulfovibrio sp. FW1012B	DFW101DRAFT_	3230
Desulfovibrio vulgaris Hildenborough	DVU	2475
Desulfomicrobiacae		
Desulfomicrobium baculatum	Dbac_	0616
Desulfobacteraceae		
Desulfatibacillum alkenivorans	Dalk_	4359
Desulfobacterium autotrophicum HRM2	HRM2_	08790
Desulfococcus oleovorans Hxd3	Dole_	0316
Desulfohalobiacae		
Desulfohalobium retbaense DSM 5692	Dret_	0312
Desulfonatronospira thiodismutans ASO3-1	DthioDRAFT_	2700
Desulfobulbaceae		
Desulfotalea psychrophila	DP	
Desulfurivibrio alkaliphilus	DaAHT2_	2006
Syntrophobacteraceae		
Syntrophobacter fumaroxidans MPOB	Sfum_	2151
Clostridia		
Peptococcaceae		
Desulfotomaculum acetoxidans DSM 771	Dtox_	2597
Desulfotomaculum reducens	Dred_	1070
C. Desulforudis audaxviator MP104C	Daud_	0550
Thermoanaerobacterales		
Ammonifex degensii KC4	Adeg_	0374
Nitrospirae		
Thermodesulfovibrio yellowstonii	THEYE_	

APPENDIX II

SUPPLEMENTARY MATERIAL OF CHAPTER 4

Figure II.1 - Sequence alignments of *Pyrococcus furiosus* soluble hydrogenases I (PF0892) and II (PF1330) γ subunit and *D. vulgaris* Hildenborough (DvH) FloxA. Conserved residues are shaded in light blue; conserved cysteines are shaded in black and marked with **C**. The FAD domain is highlighted in yellow, the NAD(P) domain is highlighted in salmon and Fe-S domain is highlighted in green.

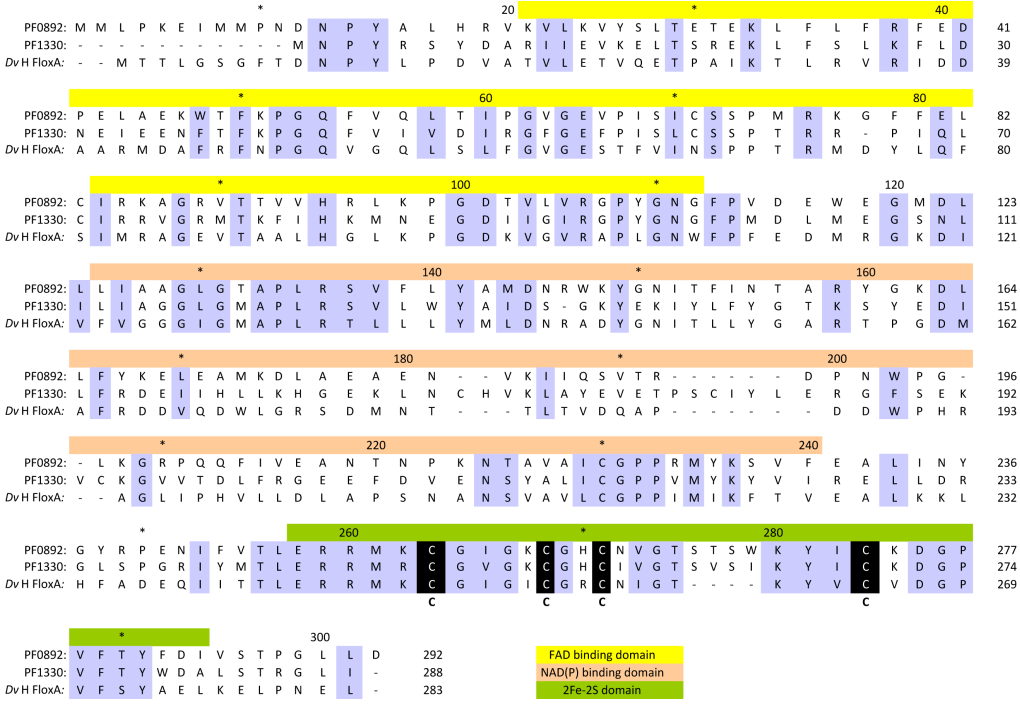


Figure II.2 - Sequence alignments of *Pyrococcus furiosus* soluble hydrogenases I (PF0891) and II (PF1329) β subunit and *D. vulgaris* Hildenborough (DvH) FloxB. Conserved residues are shaded in light blue; conserved cysteines are shaded in black and marked with **C**. The Fe-S binding domain is highlighted in pink.

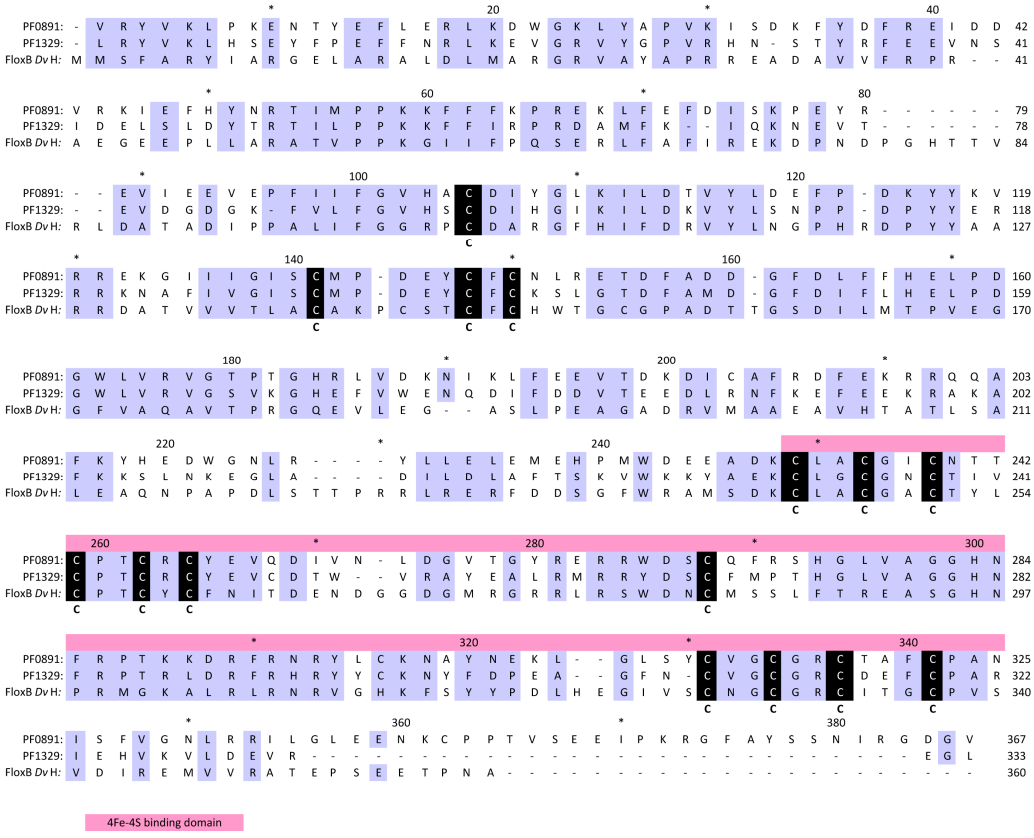


Figure II.3 - Sequence alignments of *Pyrococcus furiosus* soluble hydrogenases I (PF0891) and II (PF1329) β subunit and the FloxC domain (residues 151-494) of *D. vulgaris Hildenborough* (DvH) FloxCD. Conserved residues are shaded in light blue; conserved cysteines are shaded in black and marked with **C**. The Fe-S binding domain is highlighted in light yellow.

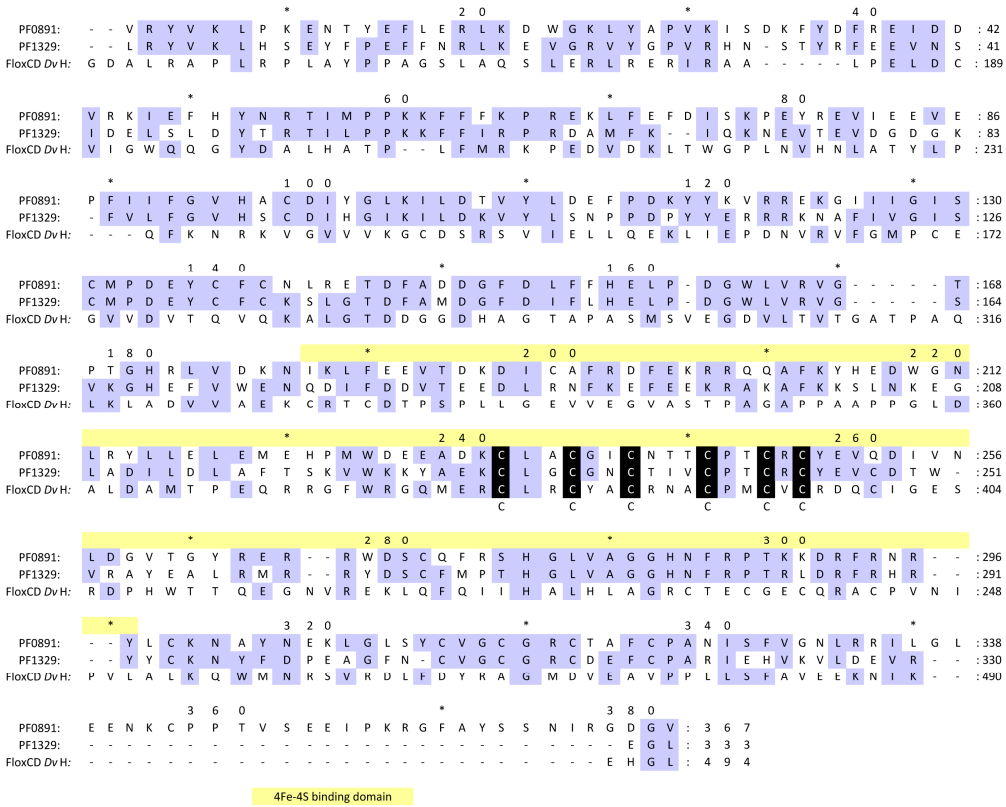


Figure II.4 - Sequence alignments of MvhD from *Methanothermobacter marburgensis* and the FloxD domain (residues 1-150) of FloxCD from *D. vulgaris* Hildenborough (*Dv*H). Conserved residues are shaded in light grey, conserved cysteines are shaded in black and marked with **C**, and MvhD domain is marked in purple.

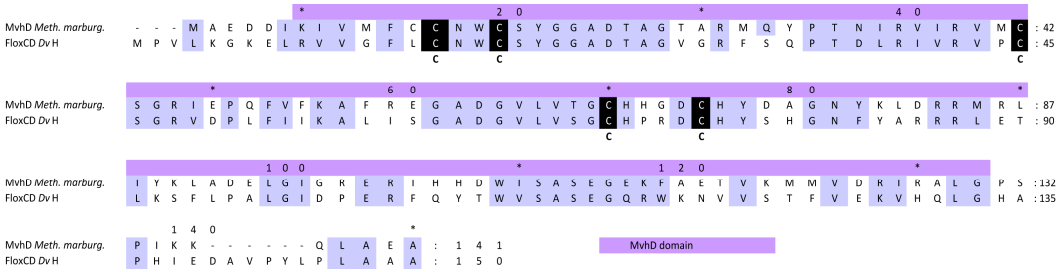
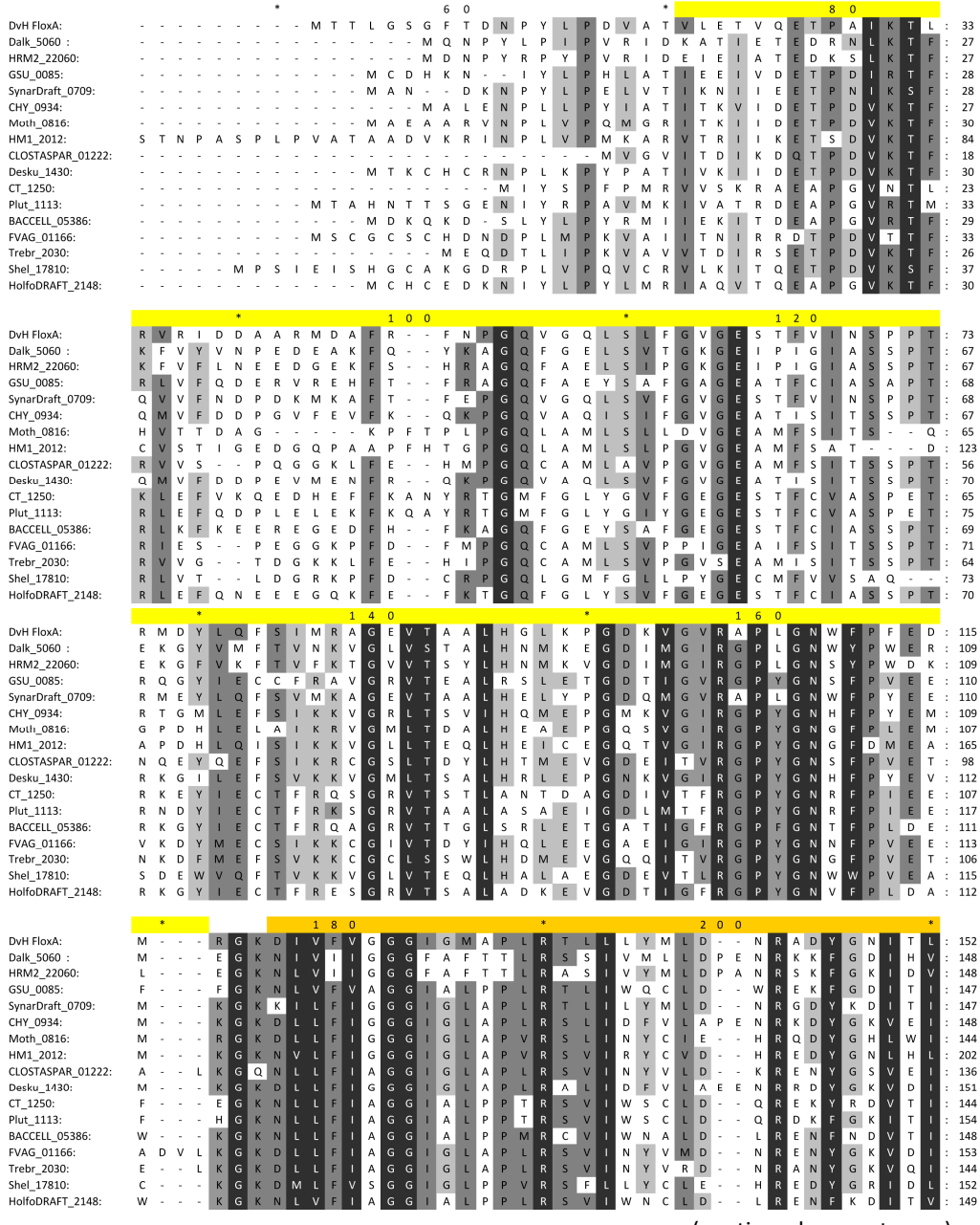


Table II. 1 – Locus tag of the *flox-hdr* operon from selected genomes. ‡ - *floxCD* fused gene; $^{\Delta}$ only *hdrA* gene present; *only *hdrL* gene present; † - *hdrBC* fused gene

	Locus	Adh	HdrC	HdrB	HdrA	FloxD	FloxC	FloxB	FloxA	Adh
Deltaproteobacteria										
Desulfovibrio vulgaris Hildenborough	DVU_	2405	2404	2403	2402	2401‡		2400	2399	2396
Desulfatibacillum alkenivorans	Dalk_				5064$^{\Delta}$	5063	5062	5061	5060	
Desulfobacterium autotrophicum	HRM2_				22100*	22090	22080	22070	22060	
Geobacter sulfurreducens	GSU		0092	0091	0090	0089	0088	0087	0085	
Syntrophorhabdus aromaticivorans	SynarDRAFT_		0715	0714	0713	0712	0711	0710	0709	
Firmicutes/Clostridia										
Carboxydothermus hydrogenoformans	CHY_	0925	0927	0929	0930	0931	0932	0933	0934	
Moorella thermoacetica ATCC 39073	Moth_		0809	0810	0812	0813	0814	0815	0816	
Heliobacterium modesticaidum	HM1_		2006	2007	2008	2009	2010	2011	2012	
Clostridium asparagiforme DSM 15981	CLOSTASPAR_		01227	01228	01226	01225	01224	01223	01222	
Desulfotomaculum kuznetsovii 17	Desku_		1436	1435	1434	1433	1432	1431	1430	
Chlorobi										
Chlorobium tepidum TLS	CT_		1245†		1246	1247	1248	1249	1250	
Pelodictyon luteolum DSM 273	Plut_		1108†		1109	1110	1111	1112	1113	
Bacteroidetes										
Bacteroides cellulosilyticus DSM 14838	BACCELL_		05392	05391	05390	05389	05388	05387	05386	
Fusobacteria										
Fusobacterium varium ATCC 27725	FVAG_		01171	01172	01170	01169	01168	01167	01166	
Spirochaetes										
Treponema brennaborense	Trebr_		2035	2036	2034	2033	2032	2031	2030	
Actinobacteria										
Slakia heliotrinireducens	Shel_		17750	17760	17770	17780	17790	17800	17810	
Acidobacteria										
Holophaga foetida DSM6591	HolfoDRAFT_		2142	2143	2144	2145	2146	2147	2148	

Figure II.5 - Sequence alignments of FloxA from the 17 genomes selected for analysis. Sequence order is as presented in Table II.1 Conserved residues are shaded in dark grey and partially conserved residues in light grey. Conserved cysteines are shaded in black and marked with C. Domains from *D. vulgaris* are highlighted in the following colors: FAD domain in yellow, NAD(P) in orange and Fe-S binding domain in green.



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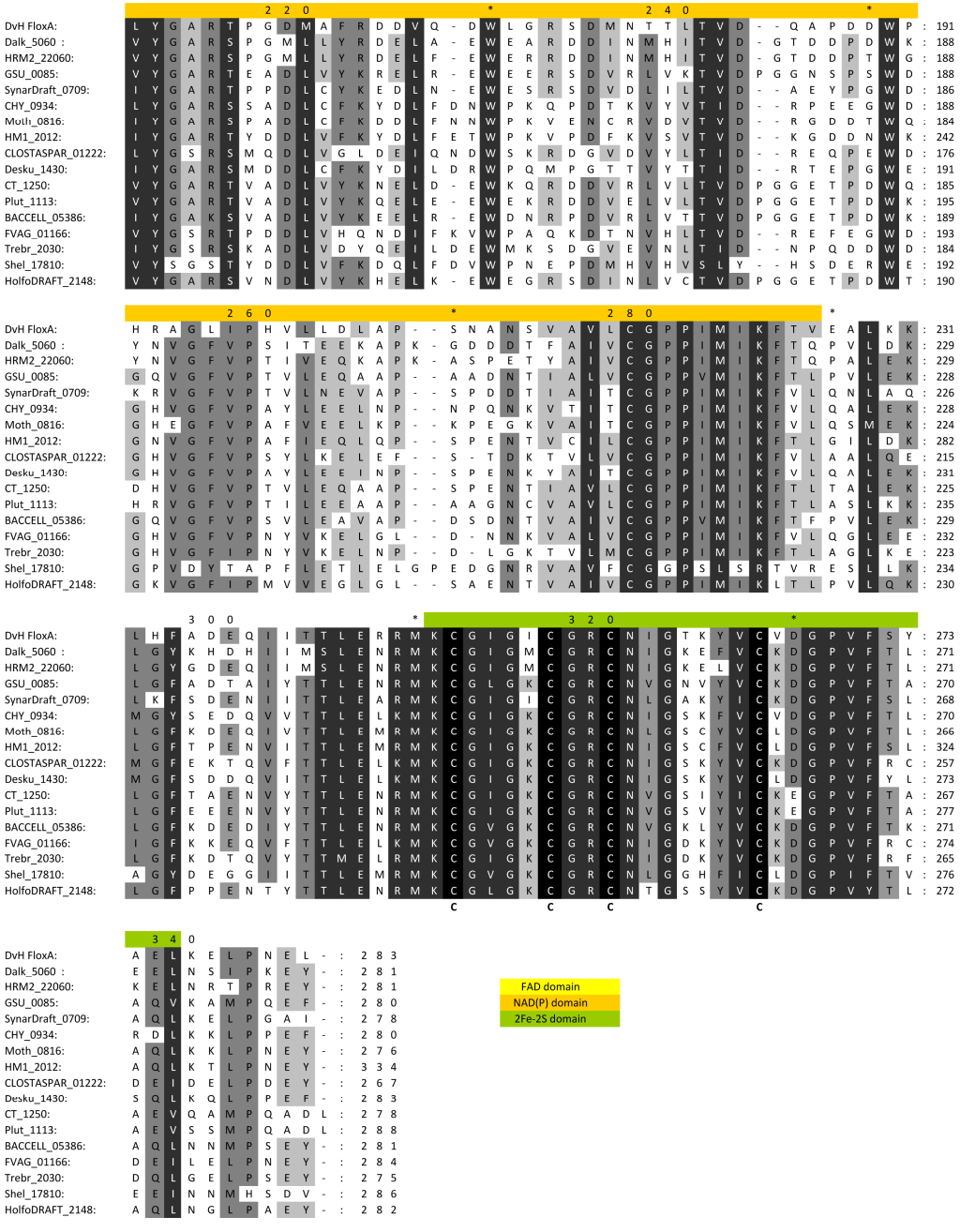
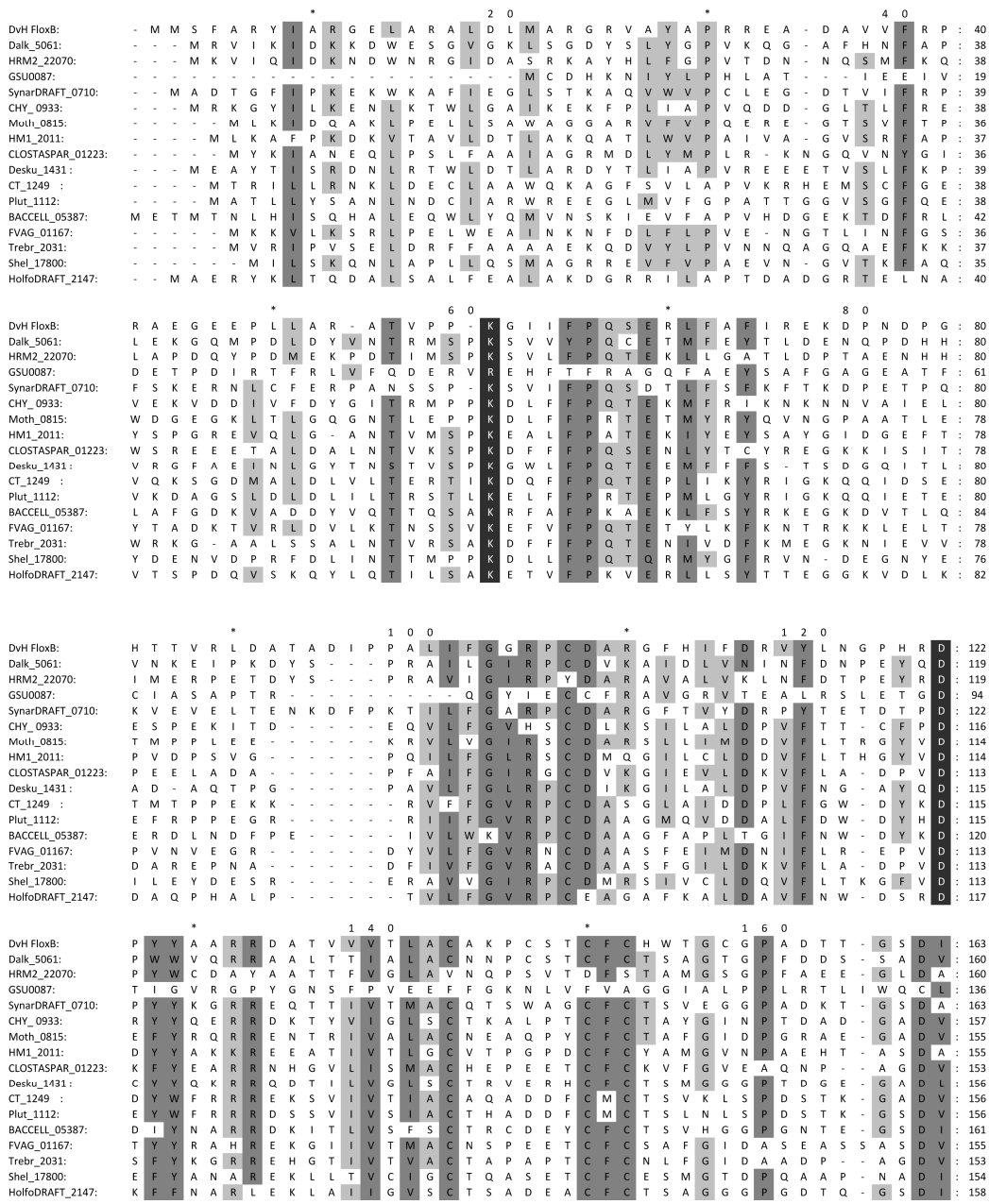

FAD domain
NAD(P) domain
2Fe-2S domain

Figure II.6 - Sequence alignments of FloxB from 17 genomes selected for analysis. Sequence order is as presented in Table II.1. Conserved residues are shaded in dark grey and partially conserved residues in light grey. Conserved cysteines are shaded in black and marked with **C.** The Fe-S binding domain from *D. vulgaris* is highlighted in pink.



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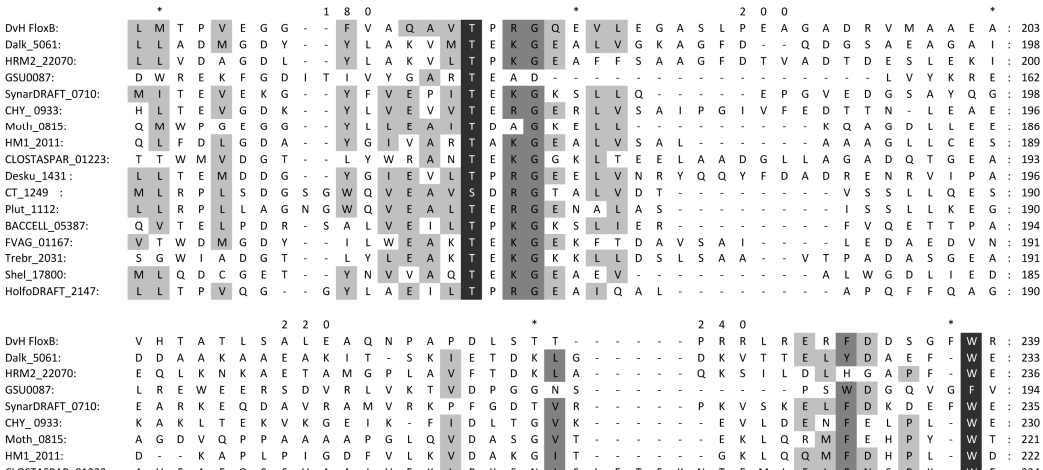

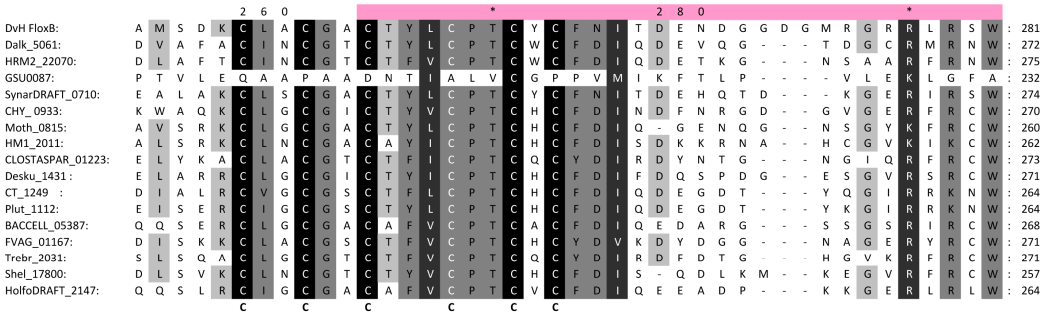

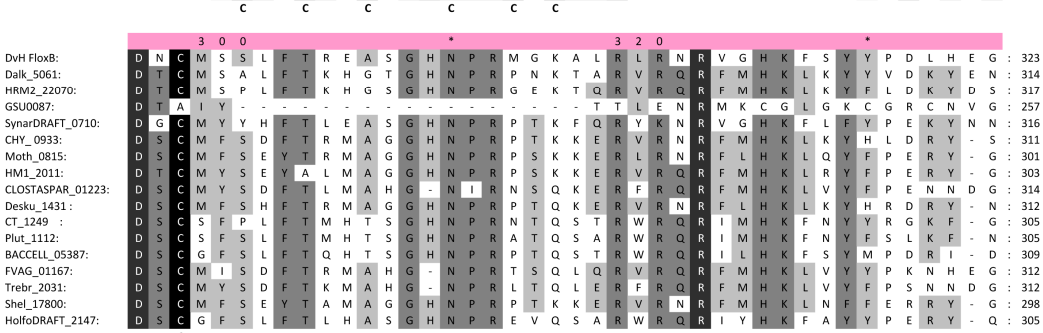

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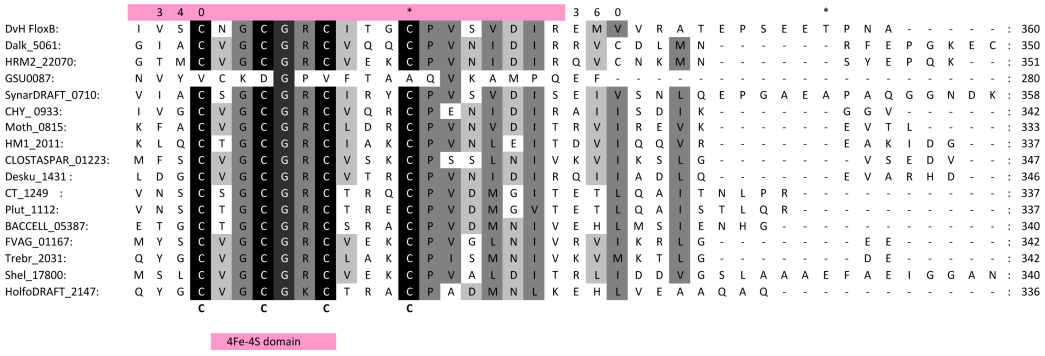
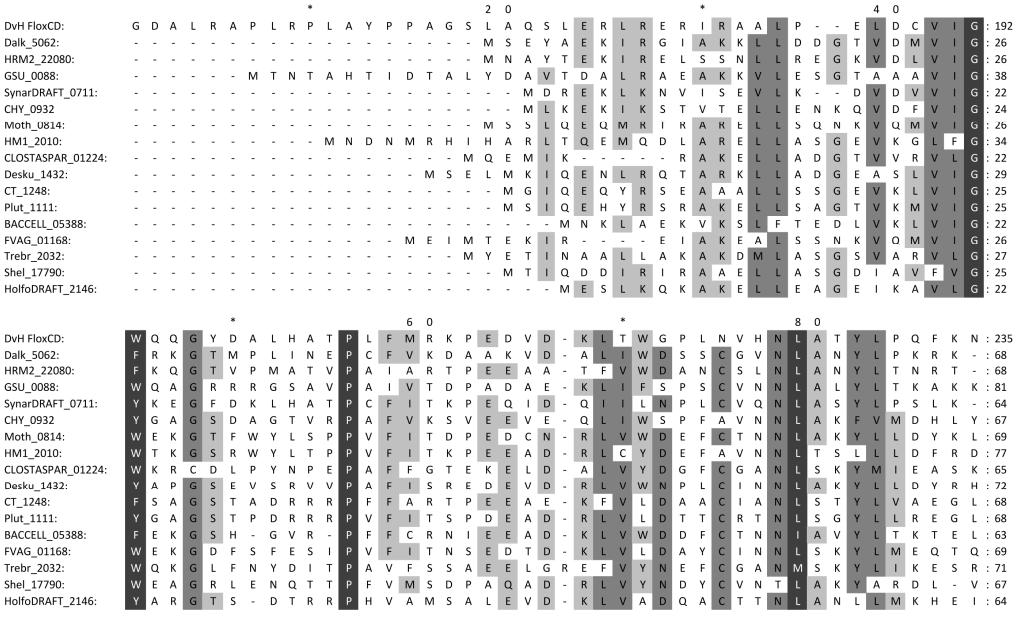


Figure II.7 - Sequence alignments of FloxC domain (residues 151-494) from *D. vulgaris* and FloxC from the other 16 genomes selected for analysis. Sequences order is as presented in Table II.1. Conserved residues are shaded in dark grey and partially conserved residues in light grey. Conserved cysteines are shaded in black and marked with **C**. The Fe-S binding domain from *D. vulgaris* is highlighted in salmon.



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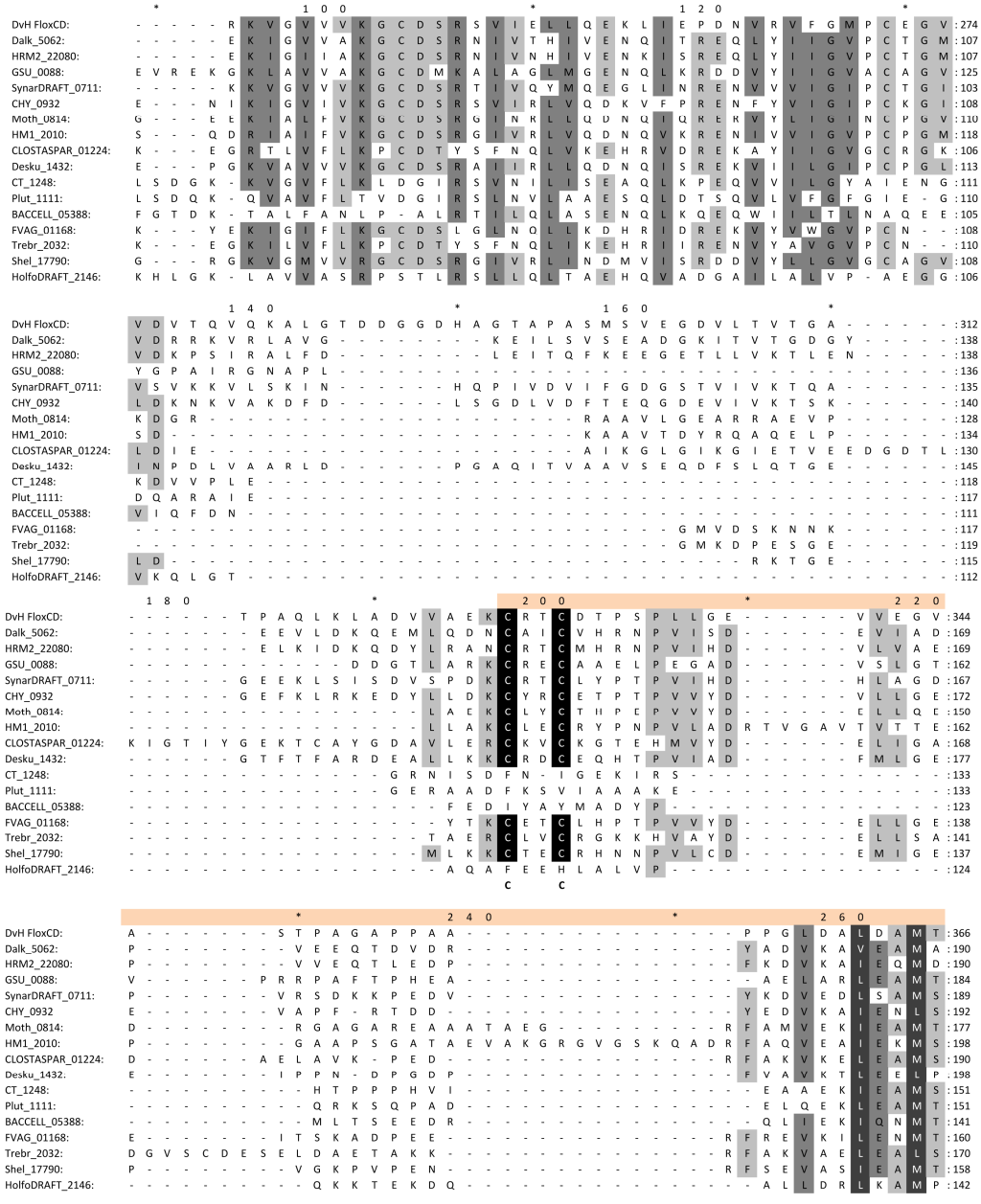

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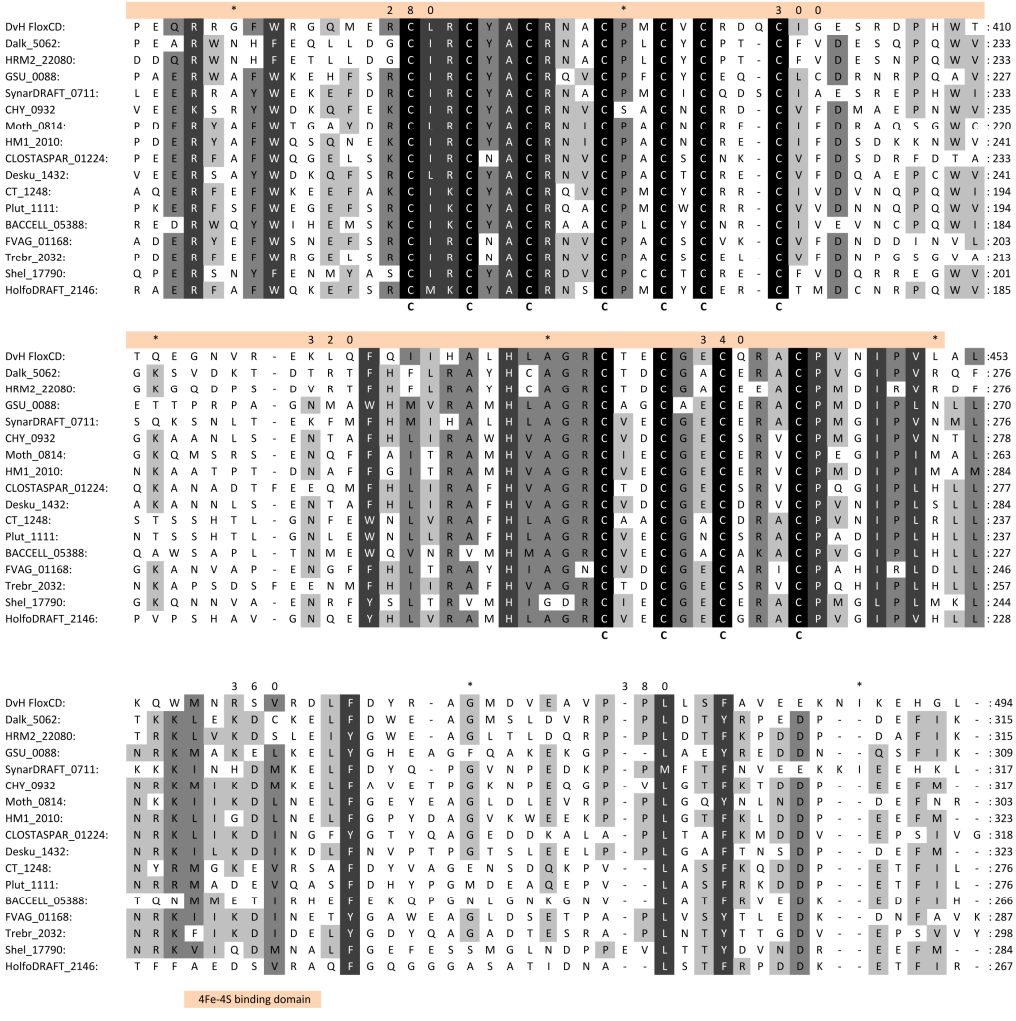
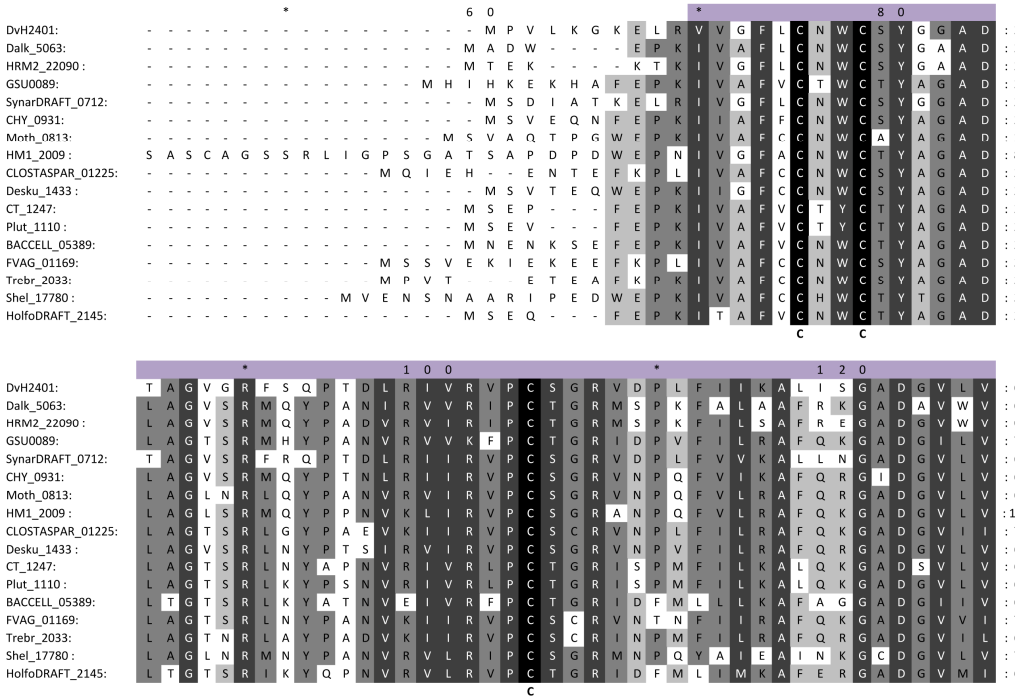

DvH FloxCD:
Dalk_5062:
HRM2_22080:
GSU_0088:
SynarDRAFT_0711:
CHY_0932
Moth_0814:
HM1_2010:
CLOSTASPAR_01224:
Desku_1432:
CT_1248:
Plut_1111:
BACCELL_05388:
FVAG_01168:
Trebr_2032:
Shel_17790:
HolfoDRAFT_2146:
4Fe-4S binding domain

Figure II. 8 - Sequence alignments of FloxD domain (residues 1-150) from *D. vulgaris* and FloxD from the other 16 genomes selected for analysis. Sequences order is as presented in Table II.1. Conserved residues are shaded in dark grey and partially conserved residues in light grey. Conserved cysteines are shaded in black and marked with **C**. The MvhD domain from *D. vulgaris* is highlighted in purple.



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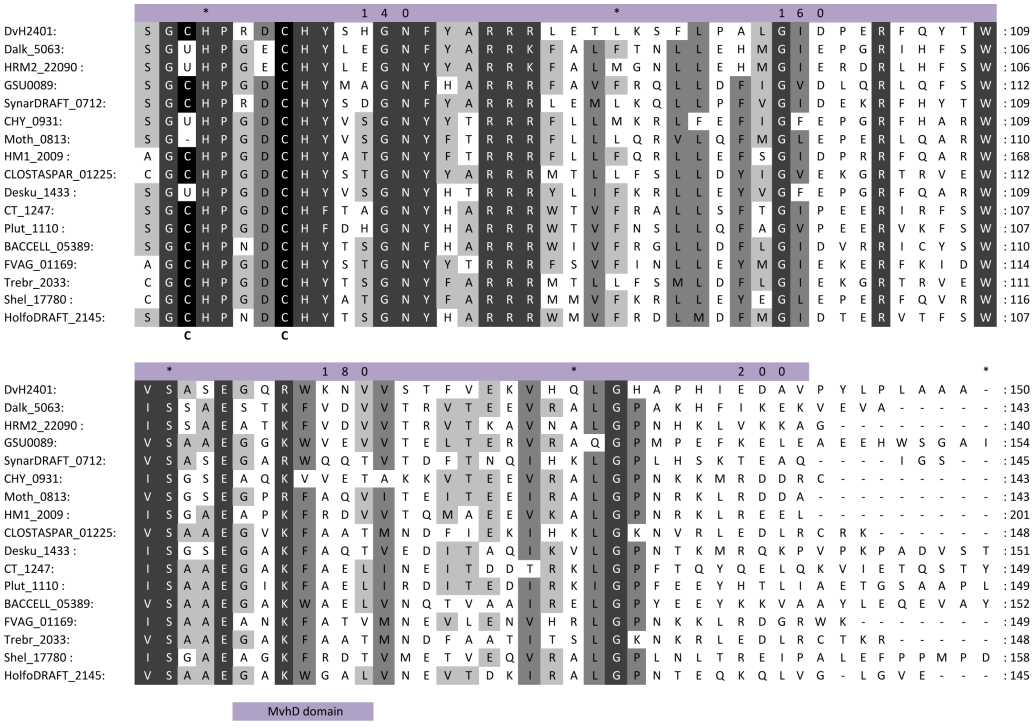
DvH2401:
Dalk_5063:
HRM2_22090 :
GSU0089:
SynarDRAFT_0712:
CHY_0931:
Moth_0813:
HM1_2009 :
CLOSTASPAR_01225:
Desku_1433 :
CT_1247:
Plut_1110 :
BACCELL_05389:
FVAG_01169:
Trebr_2033:
Shel_17780 :
HolfoDRAFT_2145:
MvhD domain

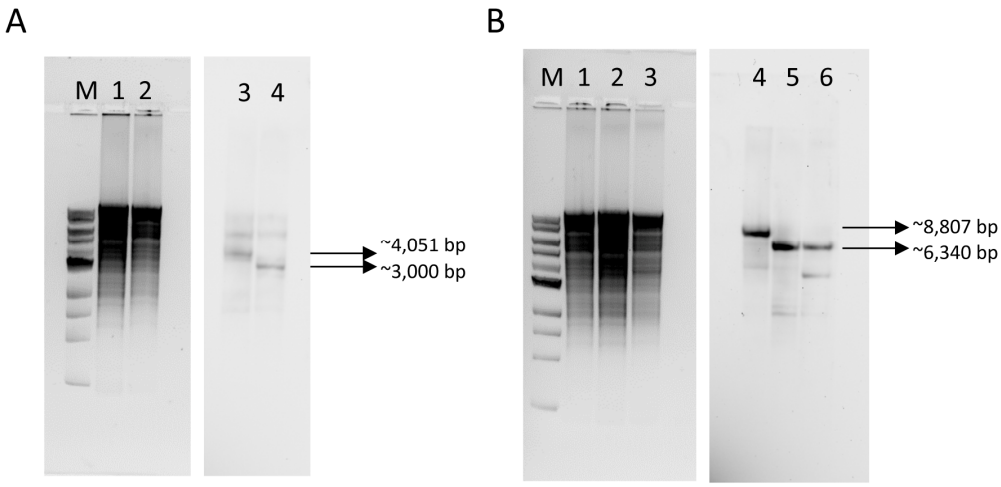

Figure II. 9 - Southern blot analysis of *flox-hdr* mutant stains. A - IPFG01 (*hdrC*::ΩKm) strain probed with *hdrC* downstream region. *Dv*H wt (lane 1) and IPFG01 (lane 2) genomic DNA's digested with *Bci*VI. Expected band sizes after detection are as follows: 4,051 bp for wt (lane 3) and 2,891 bp for IPFG01 (lane 4). B - IPFG02 (Δ*floxA*::Km) and IPFG03 (Δ*floxA*::Km) strains probed with *floxA* upstream region. *Dv*H wt (lane 1), IPFG02 (lane 2) and IPFG03 (lane 3) genomic DNA's digested with *Bci*VI. Expected band sizes after detection are as follows: 8,807 bp for wt (lane 4) and 6,340 bp for IPFG02 and IPFG03 (lane 5 and 6, respectively). DNA fragment sizes were determined by comparison with the migration of the GeneRuler 1 kb DNA ladder (Fermentas) - M.

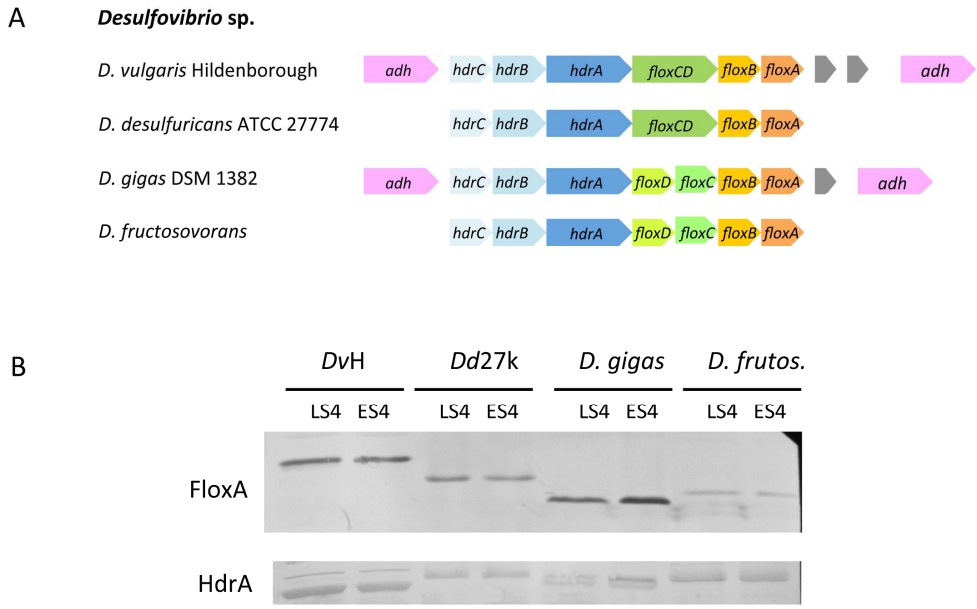

Figure II.10 - Analysis of FloxA and HdrA expression in other *Desulfovibrio* spp.. (A) – *flox-hdr* operon organization in *D. vulgaris* Hildenborough (DvH), *D. desulfuricans* ATCC 27774 (Dd27k), *D. gigas* DSM 1382 (*D. gigas*) and *D. fructosovorans* (D. frutos.); (B) - Western blots of *Desulfovibrio* spp. crude extracts using antibodies against FloxA and HdrA subunits. Cells were grown with lactate-sulfate (LS4) and ethanol-sulfate (ES4), and were harvested in the beginning of the stationary phase. The amounts for immunodetection were 25 µg of soluble crude extract for both antibodies.

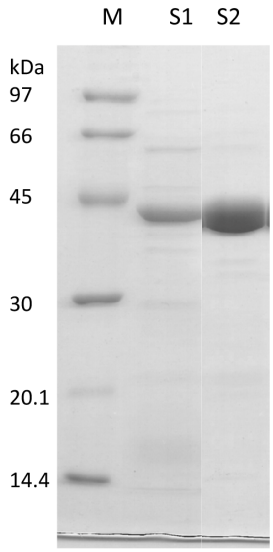

Figure II. 11 - SDS-PAGE of Adh purification fractions. S1 –Fraction from the first chromatographic step; S2 – Fraction from the second chromatographic step; M - Molecular Weight markers. The identity of the main band at 42 kDa was confirmed by Mass Spectrometry as being Adh1 (DVU2405) with a molecular weight of 41.7 kDa.